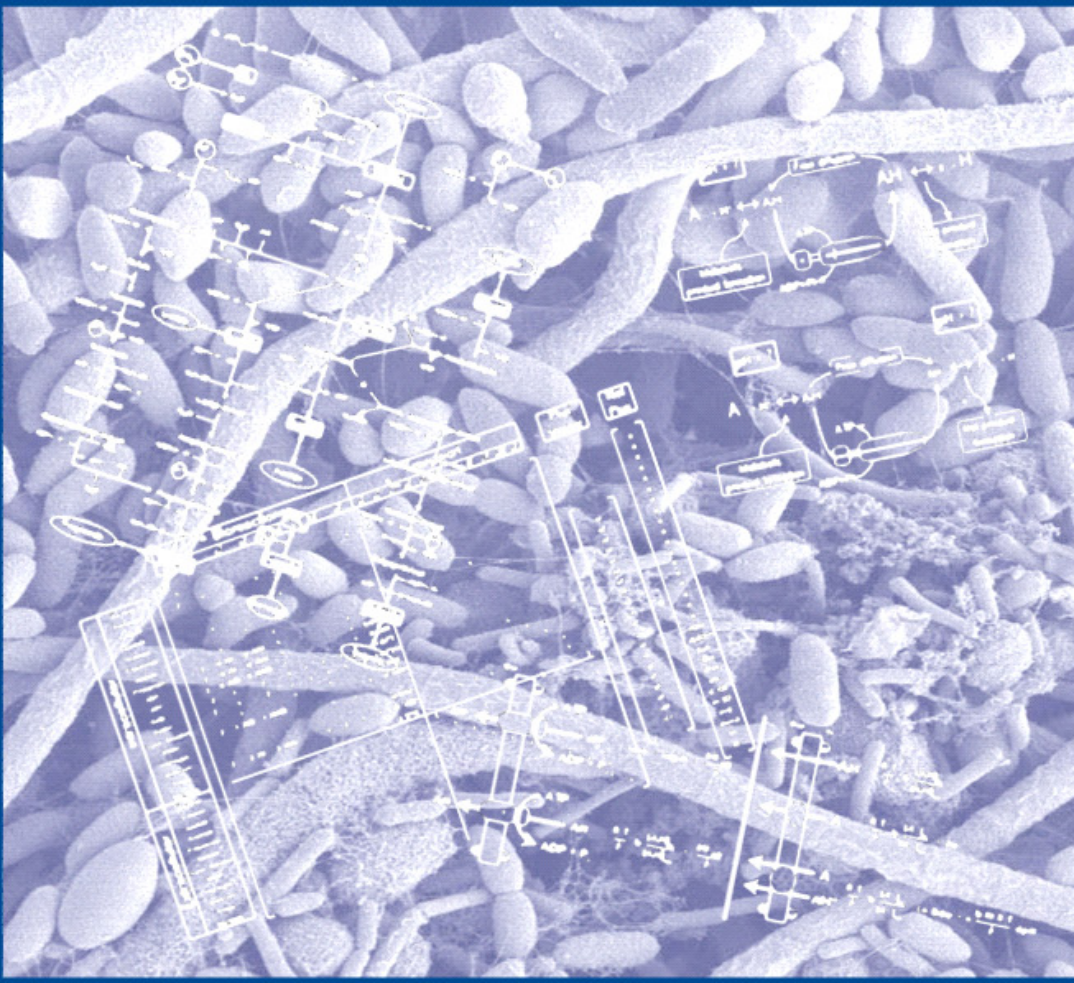
Universidade de Santiago de Compostela
Departamento de Enxeñería Química

Modelling Anaerobic Mixed Culture Fermentations



Jorge Rodríguez Rodríguez

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Departamento de Enxeñería Química

Modelling Anaerobic Mixed Culture Fermentations

Memoria presentada por

Jorge Rodríguez Rodríguez

Para optar ó grao de Doutor pola
Universidade de Santiago de Compostela

Santiago de Compostela, Xuño de 2006

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UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Departamento de Enxeñería Química

Juan Manuel Lema Rodicio, Catedrático de Enxeñería Química da Universidade de Santiago de Compostela e Robbert Kleerebezem, Profesor Asistente da Universidade Técnica de Delft (Paises Baixos),

Informan:

Que a memoria titulada "Modelling anaerobic mixed culture fermentations" que, para optar ó grao de Doutor en Enxeñería Química, Programa de Doutoramento en Enxeñería Química e Ambiental, presenta Don Jorge Rodríguez Rodríguez, foi realizada baixo a nosa inmediata dirección no Departamento de Enxeñería Química da Universidade de Santiago de Compostela.

E para que así conste, firman o presente informe en Santiago de Compostela, febreiro de 2006.

Juan M. Lema Rodicio

Robbert Kleerebezem

Esta tese foi presentada o día 2 de xuño de 2006 na Escola Técnica Superior de Enxeñería da Universidade de Santiago de Compostela ante o seguinte tribunal:

Presidente

Prof. Mark C.M. van Loosdrecht
Afdeling Biotechnologie
Technische Universiteit Delft (Paises Baixos)

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Dr. Enrique Roca Bordello
Departamento de Enxeñería Química
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Dr. Eugenio Fernández Carrasco
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Universidade de Santiago de Compostela (Campus de Lugo)

Dr. Jean-Philippe Steyer
Laboratoire de Biotechnologie de l'Environnement
Institute National de la Recherche Agronomique (Francia)

Dr. Juan Antonio Baeza Labat
Departament d'Enginyeria Química
Universitat Autònoma de Barcelona

Obtivo a calificación de ***Sobresaliente cum laude*** e a mención de ***Doutoramento Europeo***

Agradecimientos

El momento de concluir un trabajo materializado en una tesis doctoral es una buena oportunidad para reconocer la contribución de muchas personas, inseparable del trabajo en sí mismo y del doctorando. Como autor de la tesis quiero agradecer en estas páginas a todas esas personas y explícitamente a algunas de las que han dejado mayor impronta en este trabajo y también en mí mismo a lo largo de este tiempo.

Un trabajo de investigación y en particular una tesis doctoral por su carácter formativo además de investigador, es simplemente irrealizable sin una dirección experta y comprometida. La contribución de los directores de esta tesis se extendió no sólo a aspectos científicos sino también a infinidad de destrezas que se adquieren de ellos simplemente por el hecho de trabajar tantos años bajo su supervisión.

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La tesis fue realizada en el grupo de Ingeniería Ambiental y Bioprocesos de la USC, un grupo cuyos componentes han ido cambiando a lo largo de los siete años que llevo en él. Hemos vivido momentos muy agradables y aprendido mucho de muchos compañeros que han ido dejando su huella. Todos los compañeros con los que he convivido desde el principio hasta hoy merecen un agradecimiento muy especial por unos años tan agradables y por haber compartido conmigo trabajo y también diversión. Por aquí han pasado profesores, becarios, doctorandos, estudiantes, administrativos, técnicos... gracias a todos los de ahora y también a los de antes.

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Me gustaría mostrar también mi reconocimiento al Departamento de Ingeniería Química y a la Universidade de Santiago de Compostela como instituciones que han acogido este trabajo.

Quiero agradecer también a toda mi familia y en especial a mi hermana y a mi hermano. Me gustaría dedicar la tesis a mis padres, por la educación y apoyo incondicionales de que siempre he disfrutado desde que tengo conciencia y a Bárbara, por haber estado a mi lado estos años y aceptar venirse ahora conmigo a la aventura.

Santiago de Compostela, febrero de 2006.

"Si buscas resultados distintos, no hagas siempre lo mismo"
Albert Einstein

"Siempre que enseñes, enseña a la vez a dudar de lo que enseñas"
José Ortega y Gasset

"En el fondo, los científicos somos gente con suerte;
podemos jugar a lo que queramos durante toda la vida"
Lee Smolin

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Resumen

Los procesos de fermentación anaerobia vienen teniendo lugar de forma natural desde hace millones de años llevados a cabo por diversas poblaciones de microorganismos que transforman unas sustancias químicas en otras. Estos procesos de fermentación en cultivos mixtos (FCM) de microorganismos tienen lugar como parte integrante de los ciclos naturales sostenidos por el aporte continuado de energía solar al planeta. Los microorganismos involucrados obtienen energía mediante estas conversiones y de ese modo pueden sobrevivir y reproducirse, garantizando la continuidad de sus genes a lo largo del tiempo. De la misma forma que con muchos otros procesos naturales, el ser humano viene utilizando desde hace tiempo fermentaciones microbianas para desarrollar tecnologías para su propio interés. Así, el hombre aprovecha las conversiones que estos microorganismos realizan y obtiene productos valiosos o logra degradar substratos indeseados. Con esta perspectiva esta Tesis contribuye al modelado de fermentaciones anaerobias en cultivo mixto con el fin de desarrollar y mejorar tecnologías para fines diversos, entre los que se encuentran la producción de sustancias con valor de mercado o portadoras de energía o procesos de tratamiento de residuos. Esta Tesis contribuye al modelado de los procesos de fermentación en cultivos microbianos mixtos desde distintas perspectivas, cuya idoneidad depende de la aplicación y objetivos que tengan los modelos.

En el **Capítulo 1** se presentan algunos fundamentos de las fermentaciones anaerobias y se describen las biorreacciones más comunes, con una especial atención a la energética y termodinámica de las mismas. En particular, se analizan los contenidos de energía de las distintas especies químicas involucradas así como los cambios de energía a través de las diversas reacciones. Se pone de manifiesto, además, como la necesidad que tienen los microorganismos de producir sustancias muy oxidadas y de bajo contenido energético para obtener la energía metabólica necesaria para crecimiento y mantenimiento, obliga a la formación de otras sustancias de mayor contenido energético y con un menor grado de oxidación para cerrar los balances de materia y carga, así como para satisfacer las restricciones termodinámicas. Estas sustancias reducidas y de alto contenido energético son, en general, productos de interés tecnológico. En este capítulo se presentan también algunos fundamentos teóricos de modelado matemático con el fin de fundamentar y ubicar el trabajo a realizar en la Tesis. De estos fundamentos teóricos se pone de manifiesto la importancia de plantear correctamente y tener muy presentes los objetivos que se buscan a lo largo de cualquier el proceso de modelado. Se presenta una breve historia del

modelado de los procesos de digestión anaerobia, donde se puede observar como la mayoría de los trabajos realizados hasta la fecha se han centrado fundamentalmente en aplicaciones orientadas al tratamiento de aguas residuales o de residuos sólidos y no en la obtención de productos de valor. Existe un interés emergente en los procesos anaerobios de fermentación con el objetivo de obtener productos con valor de mercado, como pueden ser bioplásticos, sustancias combustibles portadores de energía como hidrógeno, metano o ciertos alcoholes, otras sustancias de interés como precursores de productos químicos, etc. Esto complementa la mera aplicación de los procesos anaerobios de fermentación para el tratamiento de corrientes residuales que venía siendo tan relevante hasta la fecha. Este interés motiva el avance y desarrollo en este campo, en el que se tenga en cuenta la mecanística de los fenómenos que rigen estos procesos, con el fin de controlar la formación de productos.

En el **Capítulo 2** de la Tesis se presenta un nuevo y prometedor enfoque para abordar el modelado de procesos de FCM, basado en realidades mecanísticas y en particular orientado hacia la predicción de la formación de productos. El cultivo mixto microbiano es considerado como si fuese un microorganismo virtual capaz de llevar a cabo las rutas metabólicas de fermentación más comunes y todo ello implantado en forma de red metabólica. Los productos de fermentación inicialmente considerados son acetato, propionato, butirato, lactato y etanol junto con hidrógeno y dióxido de carbono y se busca una relación entre la formación de unos u otros con las condiciones ambientales de operación. De este modo se desarrolla un modelo inicial que predice la formación de productos en función de variables ambientales de operación del cultivo como son el pH, la presión parcial de hidrógeno y la concentración de substrato. Para calcular los flujos de productos se procede a la optimización de la red metabólica, tomando como valor objetivo la máxima producción de biomasa, que a su vez se encuentra limitada por la disponibilidad de energía como ATP. Se trata de un problema de maximización con restricciones, definidas por el cumplimiento de los balances de materia así como de las leyes termodinámicas que obligan a que cada reacción presente un cambio de energía libre de Gibbs negativo. Las condiciones ambientales de operación impuestas afectan a la energética de las bioconversiones conducentes a los diversos productos; así se ve afectada la cantidad de ATP que se obtiene o bien el coste energético derivado de la producción de

ciertos productos. De este modo, por ejemplo, el transporte hacia el exterior de la célula de productos ácidos se vuelve energéticamente costoso a valores de pH bajos en el medio, por tener que realizarse en contra del gradiente de concentración de la forma libre del ácido. Además, la forma libre de los ácidos difunde libremente a través de la membrana celular con lo cual si se producen productos ácidos a bajo pH en el medio, éstos difunden hacia el interior de la célula cuyo pH es neutro y obligan, con el fin de mantener la homeostasis celular, a un consumo de energía para su reexpulsión. Así los productos de carácter ácido perderían rendimiento energético para la célula y en estas condiciones se favorecerá la formación de otros productos de carácter no ácido, buscando el máximo beneficio energético. La suposición del funcionamiento energéticamente óptimo por parte de los microorganismos se fundamenta en la hipótesis de que la evolución ha seleccionado a aquellos organismos capaces de aprovechar al máximo la energía disponible en el medio para su crecimiento. Los resultados obtenidos con el modelo inicial desarrollado predicen que el producto principal de la fermentación pasa a ser butirato en lugar de acetato al disminuir el pH del medio y/o aumentar la presión parcial de hidrógeno. Además para valores de pH inferiores, el etanol se convierte en el principal producto de la fermentación debido a su carácter no ácido. Este primer modelo requiere de validación experimental bajo condiciones muy bien controladas que permitan identificar procesos y parámetros y es concebido como semilla de una nueva generación de modelos mecanísticos para FCM.

El modelo desarrollado en el Capítulo 2 se plantea como un primer modelo conceptual que permita una primera estimación de los productos que se forman en FCM. En el **Capítulo 3** de la Tesis se discuten con detalle las importantes suposiciones realizadas durante el desarrollo del modelo. Se proponen además una serie de modificaciones a incorporar en el modelo que tendrán un mayor o menor impacto sobre los resultados obtenidos de su aplicación. Entre los aspectos considerados para incluir se encuentran: (i) los procesos de transferencia de materia líquido-gas (que son controlados cinéticamente) y que afectan a los valores de concentraciones en fase líquida de, por ejemplo, hidrógeno, con valores que pueden ser muy superiores a los de equilibrio; (ii) el papel del ácido fórmico como transportador de electrones ya que puede ser excretado como producto final ácido o bien descomponerse en hidrógeno y dióxido de carbono; (iii) la incorporación de otras moléculas transportadoras de electrones, además del NAD como son el FAD, Fd o el NADP y que

pueden aportar restricciones adicionales al sistema y permitir la predicción mediante el modelo de situaciones con formación de productos distintos. En este capítulo se evidencian como de especial importancia, diversos procesos controlados cinéticamente que tienen lugar en estos sistemas y no han sido considerados en la versión inicial del modelo, basada fundamente en procesos controlados termodinámicamente. Entre estos procesos controlados cinéticamente se analiza, en particular, el papel de eventuales limitaciones cinéticas inducidas por restricciones termodinámicas. Las leyes termodinámicas pueden imponer restricciones que en ocasiones obliguen a disminuir mucho la concentración de los productos de algunas reacciones para permitir su flujo, manteniendo su cambio de energía libre de Gibbs en valores negativos. Esto puede llevar a que ciertas especies intermedias en una ruta metabólica deban tomar valores de concentración extremadamente bajos, lo cual puede llevar a una situación de bloqueo cinético del flujo de la reacción. Este aspecto aparece como un fenómeno potencialmente interesante para mejorar la predicción de la formación de ciertos productos como puede ser el caso de la formación de lactato por bloqueo de las rutas metabólicas a través de acetil coenzima A. Finalmente en este capítulo se propone una hoja de ruta para la mejora de estos modelos en la que se recomiendan una serie de modificaciones. Se propone para ello un orden de prioridad para su implantación, siguiendo el criterio de implementar primero aquellas más eficientes en términos de las mejoras esperadas en el modelo por dicha modificación frente a la complejidad o grado de incertidumbre estimados que plantean.

En el **Capítulo 4** se presenta una extensión de Modelo de Digestión Anaerobia No.1 (ADM1) de la IWA para incorporar la degradación de etanol. La extensión se lleva a cabo mediante una modificación estructural en el modelo ADM1 original agregándose el proceso de consumo de etanol catalizado por un grupo específico de microorganismos. En primer lugar se lleva a cabo la implementación del ADM1 en *Matlab/Simulink* y se comprueba la ausencia de errores de programación mediante la comparación con los resultados de otras dos implementaciones en plataformas distintas, tanto en estado estacionario como en dinámico, simulando datos experimentales de un CSTR. Durante las simulaciones se identifican como etapas limitantes computacionales por un lado el cálculo numérico de la fisicoquímica del sistema, incluyendo el pH, y por otro la integración numérica del balance de materia del hidrógeno debido a su variación brusca de concentración en intervalos

cortos de tiempo (*stiff*). Se recomienda la aproximación algebraica tanto de la fisicoquímica como del balance de hidrógeno para mejorar la velocidad de las simulaciones siendo muy pequeño el error adicional introducido. Para estimar la estequiometría del proceso de degradación, así como los parámetros cinéticos de la velocidad de consumo de etanol se utilizan resultados experimentales obtenidos de la operación de un reactor anaerobio UASB-AF a escala piloto alimentado con vino diluido. Dadas las dificultades de identificabilidad por separado de la velocidad máxima de consumo y del rendimiento de biomasa, se estima, a partir del valor para glucosa, un valor del rendimiento sobre etanol considerando diferencia de generación de ATP entre estos dos substratos. El valor estimado de rendimiento tiene un efecto muy grande en el cálculo de la concentración de biomasa consumidora de etanol en estado estacionario, que es el valor asumido como valor de partida para las simulaciones. Se plantea de este modo un modelo ADM1 modificado incorporando el proceso de degradación de etanol en su estructura y utilizando los valores estimados para los nuevos parámetros. El modelo modificado se calibra con datos procedentes de varios experimentos en los que se realizan diversas sobrecargas en el reactor UASB-AF piloto. Los resultados obtenidos evidencian ciertas limitaciones en la aplicabilidad del modelo, heredadas del propio ADM1. Estas limitaciones estás asociadas a diversos factores como es la adopción de una estequiometría fija por parte del ADM1 y la versión modificada para los procesos de acidogénesis tanto de glucosa como de etanol respectivamente. La exhaustiva caracterización del influente requerida por el modelo es otra importante limitación asociada a la presencia de errores e incertidumbres en algunas concentraciones del influente. Otros factores tales como la gran sensibilidad de ciertos parámetros a pequeños cambios en sus valores son responsables de estos resultados. El modelo es, sin embargo, de gran interés para su aplicación al diseño y ajuste de controladores del proceso ya que es capaz de simular con gran detalle la dinámica de estos sistemas en situaciones de transición.

A la vista de las limitaciones recientemente reconocidas del ADM1 asociadas entre otros factores al uso de uso de estequiometrías fijas en los procesos de acidogénesis y al uso de unidades expresadas en DQO conjuntamente con molares, en el **Capítulo 5** se estudian los efectos derivados del uso de estequiometría variable sobre el comportamiento del modelo. En primer lugar el ADM1 se implementa en unidades molares para evitar el uso simultáneo

de unidades en DQO y molares y para facilitar la implantación posterior de las funciones de estequiometría variable. Para establecer las funciones de cambio de estequiometría como función de condiciones ambientales para realizar el estudio, se han utilizado parte de los resultados obtenidos con el modelo de FCM desarrollado en el Capítulo 2. Se construyen las funciones de variación de estequiometría con el fin de que durante la simulación, ésta pueda cambiar dinámicamente como función de condiciones tales como pH o concentración de hidrógeno. Los resultados del modelo FCM predecían un cambio de acetato a butirato como producto principal al disminuir el pH y/o aumentar la concentración de hidrógeno disuelto. Con el fin de integrar estos resultados del modelo desarrollado en el Capítulo 2 en el ADM1 de forma compatible, se ha utilizado únicamente el *ratio* obtenido entre estos dos productos de fermentación que aunque cambia dinámicamente mantiene constante la suma total de carbono en ambos productos y permite cerrar el balance de DQO mediante la mayor o menor producción de hidrógeno. De este modo los coeficientes estequiométricos del catabolismo de glucosa se calculan en cada instante. Al realizar la comparación dinámica entre el modelo ADM1 estándar y el implementado con estequiometría variable no aparecen apenas diferencias en los resultados de la simulación de una sobrecarga fuerte cuando se trata de un sistema metanogénico. Esto se atribuye a que la etapa acidogénica de fermentación de glucosa no limita el proceso con lo que la capacidad de eliminación de DQO permanece prácticamente inalterada. Por otro lado si se realiza la comparación en un sistema de digestión anaerobia en dos etapas, siendo la primera un reactor acidogénico sin microorganismos metanogénicos, aparecen importantes diferencias en la composición del efluente de este primer reactor, ya que la presión parcial de hidrógeno es mucho mayor. El efluente del reactor acidogénico entra en el metanogénico donde la eliminación de DQO global predicha por ambos modelos es nuevamente equivalente ya que la conversión de los productos de fermentación de glucosa hasta acetato no es una etapa limitante del proceso frente a la metanogénesis. Estos resultados sugieren que la incorporación de una estequiometría variable no afecta las predicciones del modelo de un modo relevante en sistemas metanogénicos pero resulta necesaria para extender la aplicabilidad del ADM1 a sistemas anaerobios no metanogénicos ya que la predicción de productos de fermentación es totalmente diferente. Se podría plantear también la posibilidad de agrupar los procesos acidogénicos y acetogénicos en un sólo proceso con estequiometría variable en función de las condiciones ambientales.

En el **Capítulo 6** se presenta un método estadístico para la caracterización estructural de modelos que puede ser aplicado también para la reducción o simplificación de los mismos. El método consiste en la aplicación de la técnica de análisis de componentes principales (ACP) a datos experimentales previamente filtrados y normalizados con el fin de determinar el número mínimo de procesos de conversión que han de ser considerados en un modelo para retener un porcentaje deseado de la variabilidad de esos datos. Esta técnica se puede englobar dentro de los llamados métodos de caracterización estructural *a priori* como apoyo en el proceso de desarrollo de modelos candidatos, en concreto en el planteamiento de su estructura a partir de datos experimentales. Los modelos candidatos se deberán plantear con al menos el número mínimo de procesos que se hayan estimado como necesarios para poder reproducir el nivel de variabilidad deseado para un caso particular. Esta técnica no define de por sí cuales son los procesos a considerar sino sólo el número necesario de éstos. Los procesos tendrán que ser definidos a partir de un conocimiento experto así como de la información obtenida de los componentes principales, que están relacionados linealmente con la estequiometría de los procesos buscados. Frecuentemente sin embargo, la extracción de ésta información de los mismos no es sencilla. Además, la técnica ACP propuesta puede ser aplicada sobre datos simulados generados mediante un modelo de complejidad alta con el objetivo de reducirlo o simplificarlo en términos de su número de procesos. Se podrá plantear un modelo más simple que el original en los casos en que la variabilidad de los datos simulados pueda ser retenida por un número de procesos menor que los presentes en el modelo complejo. Del mismo modo que lo sucedido anteriormente, se necesita un conocimiento experto del sistema para extraer la información implícita de los componentes principales obtenidos y para determinar de ese modo la relación de los procesos más adecuados a considerar en el nuevo modelo reducido. En este caso la existencia de una estructura más compleja previa, sirve de ayuda adicional para definir estos procesos. La técnica ACP presentada se aplica al análisis de los datos experimentales obtenidos en diversos experimentos de sobrecarga en un reactor UASB-AF y en un CSTR a escala piloto y laboratorio, alimentados con agua sintética e industrial respectivamente. Los resultados obtenidos indican que son suficientes cuatro procesos para retener casi completamente (en más de un 95 %) la variabilidad de los datos experimentales. De esto se concluye que un modelo con cuatro procesos debería ser capaz de reproducir datos experimentales generados bajo condiciones similares de

operación. Finalmente la técnica ACP se aplica también al tratamiento de datos simulados mediante el ADM1 con el fin de analizar si un modelo más sencillo podría reproducir su variabilidad. Se concluye de nuevo que cuatro procesos relevantes son suficientes para retener casi completamente (en más de un 95 %) la variabilidad de los datos y que por lo tanto resulta factible la simplificación del ADM1 en un modelo de solamente cuatro procesos para simular experimentos en condiciones y con aguas residuales de características similares.

Resumo

As bioconversións anaerobias ocorren na natureza dende hai miles de anos e xogan un importante papel nos ciclos globais sostidos polo aporte continuado de enerxía solar ó planeta. Estes procesos son efectuados por microorganismos que obteñen enerxía ó realizaren estas conversións e que, en ausencia dun aceptor de electróns externo, realizan fermentacións para converter os substratos orgánicos. Do mesmo xeito que con moitos outros procesos naturais, os humáns veñen utilizando as fermentacións en cultivos mixtos (FCM) microbiáns para desenvolver tecnoloxías para o seu interese. Baixo esta perspectiva, esta Tese contribúe ó modelado de fermentacións anaerobias en cultivo mixto coa fin de desenvolver tecnoloxías para diversos propósitos, incluíndo o tratamento de augas residuais e a producción de sustancias químicas de valor ou alto contido enerxético.

No Capítulo 1 preséntanse os fundamentos das fermentacións anaerobias, cunha especial atención a enerxética dos mesmos. Analízanse os fluxos de enerxía a traveso das diversas sustancias involucradas nas bioconversións, nas que a producción de sustancias reducidas e de alto contido enerxético é consecuencia da necesidade de pechar os balances de masa, carga e de cumprir coas leis termodinámicas. Neste capítulo preséntanse asemade algúns fundamentos de teoría de modelado coa fin de aportar perspectiva ó traballo a desenrolar na Tese e de onde se pon de manifesto que os obxectivos para os que se crean os modelos deben estar sempre presentes no proceso de modelado. Inclúese tamén unha breve historia do modelado dos procesos de dixestión anaerobia, amosándose como a maioría dos traballos de modelado se centraron no tratamento de augas residuais. O interese emerxente dos procesos anaerobios de FCM para obter productos con valor de mercado fronte ó mero tratamento de augas é unha das principais motivacións para o desenvolvemento desta área de coñecemento.

No Capítulo 2 preséntase un novo e prometedor enfoque para o modelado de FCMs buscando a predicción dos productos de fermentación. Os productos considerados son acetato, propionato, butirato, lactato e etanol así como hidróxeno e dióxido de carbono. O cultivo mixto microbián aproxímase como un microorganismo virtual coas rutas metabólicas de fermentación máis comúns implantadas nunha rede metabólica. Desenrólase un modelo inicial para predecir os productos de fermentación de glucosa en estado estacionario que se obteñen baixo diferentes condicións ambientais de pH, presión parcial de hidróxeno e

concentración de substrato. Os fluxos de productos calcúlanse mediante unha optimización da rede metabólica maximizando a producción de biomasa (limitada pola cantidade de enerxía como ATP) cumpríndose os balances de materia e as leis termodinámicas. As condicións ambientais afectan á enerxética das conversións cara os diversos productos e polo tanto á cantidade de ATP que se obtén delas. Este óptimo aproveitamento enerxético baséase na hipótese de que a evolución ten seleccionado aqueles microorganismos capaces de aproveitar o máximo de enerxía para o seu crecemento. O modelo desenrolado predice un cambio de acetato a butirato como producto principal da fermentación cando o pH diminúe e/ou a presión parcial de hidróxeno aumenta; a pH baixos o etanol aparece como o principal producto. Este primeiro modelo precisa dunha validación experimental baixo condicións ben controladas e foi concibido como semente dunha nova xeración de modelos mecanísticos para FCMs.

O modelo desenrolado no Capítulo 2 considérase como un primeiro modelo conceptual que permite obter unha primeira estimación dos productos formados en FCM. As importantes suposicións realizadas durante o desenvolvemento do modelo son discutidas con detalle no Capítulo 3. Propóñense unha serie de melloras máis urxentes e discútese o seu potencial de mellora do modelo. Entre os aspectos a ser resoltos atópanse a consideración dos procesos de transferencia de materia líquido-gas (controlados cinéticamente); o papel do ácido fórmico como transportador de electróns; a incorporación doutros transportadores de electróns, engadindo restricción ó sistema para mellorar a predicción de productos, etc. De especial importancia son diversos procesos cinéticos, non incluídos nesta versión do modelo, e en particular eventuais limitacións cinéticas inducidas por restricción termodinámicas que se estudian en máis detalle. As leis termodinámicas impoñen restricción que poden obrigar a diminuír a concentración de certas especies intermedias ata valores extremadamente baixos impedindo cinéticamente o fluxo de reacción. Isto foi identificado como un fenómeno de potencial interese na predicción da formación de certos productos. Finalmente neste capítulo proponse unha folla de ruta para a mellora dos modelos de FCMs, considerando primeiramente aquelas modificacións máis eficientes en termos de mellora esperada no modelo fronte á complexidade ou grao de incertidume da súa implantación.

No Capítulo 4 preséntase unha extensión do Modelo de Dixestión Anaerobia No.1 (ADM1) da IWA coa fin de incluír o tratamento de augas residuais con etanol. A extensión realízase mediante unha modificación estructural no modelo incorporando o proceso de degradación de etanol catalizado por un grupo de microorganismos específico. Primeiramente o ADM1 estándar foi implantado en *Matlab/Simulink* e validado fronte a erros de programación mediante a comparación con implantacións noutras plataformas. O cálculo numérico do pH xunto coa integración do balance de materia do hidróxeno foron identificadas como dúas etapas limitantes computacionais. Para estimar a estequiometría da degradación de etanol así como os parámetros cinéticos do consumo de etanol, utilizáronse resultados experimentais obtidos da operación dun reactor anaerobio UASB-AF a escala piloto, alimentado con viño diluído. Prantéxase un ADM1 modificado, engadindo o proceso de degradación de etanol na estructura do ADM1 estándar e cos valores estimados para os novos parámetros. O modelo modificado calíbrase con datos de varios experimentos de sobrecarga e os resultados obtidos evidencian certas limitacións, consecuencia de diversos factores, como a adopción dunha estequiometría fixa no ADM1, a detallada caracterización do influente requirida así como a alta sensibilidade de certos parámetros. Este modelo é así mesmo de grande interese de cara ó control do proceso xa que é capaz de reproducir con detalle a dinámica destes sistemas en situacións de transición.

Tendo en conta as limitacións recoñecidas no ADM1 polo uso de estequiometrías fixas nos procesos de acidoxénese, no Capítulo 5 realízase un estudio do efecto do uso de estequiometría variable no seu comportamento. En primeiro lugar o ADM1 é convertido a unidades molares para eliminar algúns erros recentemente reportados e para facilitar a implantación da estequiometría variable. En base ós resultados obtidos co modelo de FCM desenrolado no Capítulo 2, xéranse as funcións de variación da estequiometría. Estes resultados predecían un cambio de acetato a butirato como producto principal cando o pH diminúe e/ou a concentración de hidróxeno aumenta. Estas funcións calculan dinamicamente os coeficientes estequiométricos do catabolismo de glucosa en función do pH e da concentración de hidróxeno. Non aparecen diferencias importantes nos resultados da comparación do modelo estándar co de estequiometría variable na simulación dun sistema metanoxénico, atribuído elo a que a acidoxénese no é limitante no proceso. Doutra banda, na simulación de dixestión anaerobia en dúas etapas aparecen importantes

diferencias na composición do efluente do reactor acidoxénico. Estes resultados suxiren que é necesaria a consideración dunha estequiometría variable para estender a aplicación do ADM1 a sistemas non metanoxénicos. Suxírese tamén a posibilidade da agrupación dos procesos acidoxénicos e acetoxénicos nun só proceso de estequiometría variable.

No Capítulo 6 desenvólvese unha metodoloxía para a caracterización estructural e reducción de modelos. O método consiste na aplicación da análise de compoñentes principais (ACP) a datos experimentais coa fin de determinar o número de procesos de conversión que poden reter unha porcentaxe desexada da variabilidade do sistema. Isto considérase como un método de caracterización estructural *a priori* para axudar á proposta de modelos candidatos a partires de datos experimentais. Os modelos candidatos deben ter o número de procesos necesario para reproducir o nivel de variabilidade desexado do sistema. Estes procesos terán que ser definidos a partires dun coñecemento experto así como da información obtida dos compoñentes principais. Ademais disto, a técnica ACP pode aplicarse a datos simulados xerados mediante modelos de alta complexidade buscando a súa reducción ou simplificación. Poderase prantexar un modelo máis simple no caso en que a variabilidade dos datos simulados poida ser reproducida por un número de procesos menor que os presentes no modelo complexo. De novo o coñecemento experto xunto coa información obtida dos compoñentes principais serve de axuda para determinar os procesos no novo modelo reducido. A técnica ACP foi aplicada a datos experimentais obtidos de varios experimentos de sobrecargas nun reactor UASB-AF e nun CSTR alimentados con auga sintética e industrial respectivamente. Os resultados obtidos indican que catro procesos son suficientes para reter case que completamente a variabilidade dos datos. Polo tanto un modelo con catro procesos debería ser capaz de predecir datos experimentais xerados en condición similares. Por outra banda o método ACP foi aplicado tamén a datos simulados xerados co ADM1 concluíndose que de novo catro procesos son suficientes para reproducir case que completamente (en máis dun 95 %) a variabilidade dos datos simulados.

Summary

Anaerobic bioconversions have been occurring in nature for millions of years and play an important role in the global cycles as driven by solar energy and conducted by microorganisms that harvest energy from these conversions. In absence of an external electron acceptor the anaerobic microorganisms rely on fermentation processes for conversion of organic substrates. As for many other microbial processes, humans have made use of these so called mixed culture fermentation (MCF) processes to develop technologies. This thesis contributes to the modelling of anaerobic mixed culture fermentations aiming for the development of technologies for multiple purposes, including wastewater treatment processes and production of chemicals or energy carriers.

The fundamentals of anaerobic fermentations, with special attention to their energetics, are presented in Chapter 1. The bioenergetic implications of the different species involved in the bioconversions are presented, showing how the production of reduced high-energy compounds is necessary to accomplish with thermodynamic laws and mass and redox balances. A general background of modelling theory is also presented in this chapter to support the work described in this thesis. A brief history of the modelling of anaerobic digestion is presented as well, showing how most of the modelling work focused on wastewater treatment. The emerging interest of anaerobic MCFs to obtain valuable chemical products instead of only for waste treatment purposes appears as a major motivation for further development in this field.

A new and promising approach for developing mechanistic models of MCFs, aiming at the prediction of product formation as a function of the environmental conditions of cultivation, is presented in Chapter 2. The organic end products of the MCF process considered are acetate, propionate, butyrate, lactate, ethanol and biomass with hydrogen and carbon dioxide as by-products. The mixed culture microbial population is assumed as a virtual microorganism in a metabolic network that conducts the most typical fermentation pathways. An initial model was developed to predict the product spectrum obtained in steady state by fermentation of glucose under different environmental conditions of pH, partial pressure of hydrogen and substrate concentration. The fluxes of products are calculated by optimisation of the metabolic network, in terms of maximum biomass growth (limited by energy as ATP), while fulfilling thermodynamic laws and mass balances. The environmental conditions affect the energetics of the conversions towards the diverse

products and consequently the amount of ATP yielded. This optimum energetic performance is based on the hypothesis that evolution has selected those microbial species that most efficiently harvest energy for growth. The model developed predicts a shift from acetate to butyrate as main fermentation product when pH decreases and/or hydrogen pressure increases, while ethanol is predicted as main product at lower pH values. The model requires experimental validation under well controlled conditions and it was conceived as the seed of a new generation of mechanistic models for MCFs.

The model developed in Chapter 2 should be regarded as a first, conceptual model that allows for obtaining a first estimate of the products formed in MCF processes. However, during model development major assumptions and simplifications were made. These aspects of the MCF model are extensively discussed in Chapter 3. The most important and urgent add-ins to the model are also proposed and their potential for model improvement is discussed in this chapter. Among the issues to be addressed are the incorporation of liquid-gas mass transfer processes (kinetically controlled); the role of formic acid as electron carrier; incorporation of additional electron carriers that can add more constraints to the system for a more realistic products prediction, etc. Of particular importance are certain kinetic processes not currently included in the model, and among them, kinetic limitations induced by the thermodynamic constraints are studied in more detail. Thermodynamic laws impose restrictions that can decrease certain intermediate concentrations down to extremely low values creating kinetically unfeasible fluxes. These phenomena are identified of potential interest for the prediction of product formation. Finally in this chapter a route plan for improvement of MCF models is proposed, considering first the most efficient modifications in terms of expected improvements of the model output versus complexity of implementation.

The general purpose IWA Anaerobic Digestion Model No.1 (ADM1) is extended in Chapter 4 to include the treatment of ethanolic wastewaters. This is achieved by incorporating ethanol degradation process in the model, catalyzed by an independent microbial group. First, the standard ADM1 is implemented in *Matlab/Simulink* and validated for programming errors by comparison with implementations in other platforms. The numeric calculation of pH and the integration of the hydrogen mass balance are identified as two important computational bottlenecks. Experimental results obtained from the operation of

a pilot scale UASB-AF anaerobic reactor, treating diluted wine, are used to estimate the stoichiometry of ethanol degradation, as well as the kinetic parameters of the ethanol uptake rate. A modified ADM1 is built-up by addition of the ethanol degradation process to the standard ADM1 structure with the new estimated parameters. The modified model is calibrated using data from several overload experiments and the results obtained demonstrated certain limitations, due to the fixed stoichiometry utilized, the detailed influent characterisation required and the high sensitivity of certain parameters. This model appears as very interesting to be applied for control purposes, since it reproduces properly the dynamics of these systems under transient conditions.

Considering the limitations of using a fixed stoichiometry approach in the acidogenic processes recognised in ADM1, the effect of using a variable stoichiometry on the model performance is studied in Chapter 5. The standard ADM1 was first converted into molar units, to avoid some errors reported and to facilitate the subsequent implementation of a variable stoichiometry. Based on the results obtained from the MCF model developed in Chapter 2, that predict a shift from acetate to butyrate as the major product at decreasing reactor pH and/or increasing hydrogen concentration, variable stoichiometry functions are built up. These functions return dynamically the ADM1 stoichiometry coefficients of the catabolism from glucose as a function of the operational pH and the hydrogen concentration. The results of the standard versus the variable stoichiometry ADM1 showed that for methanogenic systems no difference appears in the process performance simulated, since the acidogenesis is not the rate limiting process. On the other hand when a two step anaerobic digestion process is simulated, important differences appear especially in the product composition of the acidogenic reactor. These results suggest that a variable stoichiometry approach is required to extend the application of ADM1 to non methanogenic systems. A lumping of acidogenic and acetogenic processes in ADM1 into a sole variable stoichiometry process is furthermore suggested.

A methodology for structural characterisation and model reduction is developed in Chapter 6. The method consists of the application of principal component analysis (PCA) to experimental data, in order to assess the number of conversion processes required to retain a desired percentage of the data variability. This can be considered as an *a priori* structural characterisation method that assists the modeller to generate candidate model

structures from experimental data. A candidate model should have the number of processes required to reproduce the desired level of variability of the system. The characteristics of these processes must be defined by the modeller's expertise and the information extracted from the principal components obtained. In addition to this, the PCA can be applied also to simulation data generated by complex models, aiming at their reduction or simplification. A simpler model can be set up if the simulated data variability can be reproduced by a lower number of processes than those present in the complex model. Again, the modeller's expertise and the principal components provide guidelines for the definition of the reduced model processes. The PCA methodology is applied to experimental data from overload experiments in a UASB-AF and a CSTR reactor treating synthetic and industrial wastewater respectively. The results obtained indicate that only 4 processes can retain almost completely the data variability and a four-process model should be therefore able to reproduce experimental data generated under similar conditions. Moreover the PCA method was applied to simulation data generated by the ADM1, obtaining that also 4 processes are enough to reproduce almost completely (more than 95 %) the data variability.

Chapter 1

GENERAL INTRODUCTION

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Contents

Abstract

This chapter provides background for the work developed in this thesis. Anaerobic fermentations are introduced with a brief explanation of the most important fermentation pathways, their energetics and ecological issues. General aspects of mathematical modelling are presented and a brief review of the state of the art in modelling anaerobic bioprocesses. The fundamentals of metabolic modelling tools are also introduced. An outline of the contents of this thesis is finally presented.

1.1. Anaerobic fermentations

The production of chemicals from renewable resources (e.g. carbohydrates from agro industries) and particularly from waste streams, is emerging as an important biotechnological application towards a sustainable society. These chemical products from fermentative processes gain interest not only for energetic purposes (Claassen *et al.*, 1999), given the currently increasing cost of non-renewable energy carriers, but also for production of useful chemical products. Methane production from anaerobic digestion, production of hydrogen and alcohols as ethanol or butanol from fermentation have been intensively investigated (McCarty and Smith, 1986; Benemann, 1996; Dürre, 1998). Other fermentation products are also building blocks for chemical or biological production of polymers, like polyhydroxyalkanoates (PHA) from volatile fatty acids (Reis *et al.*, 2003) or used as additives in the food industry as alcohols or lactic acid.

From a technological point of view, fermentative bioprocesses can be conducted using pure cultures of a certain microorganism, known to carry out certain conversions leading to a product of interest. This occurs most likely in industrial fermentation processes where high value products and high costs are assumed, derived from the use of sterile conditions and pure substrates, to avoid contaminations of the culture. On the other hand, mixed culture fermentations are carried out by more stable mixed microbial populations, as typically found in nature, and present much lower costs, but still much development is required to overcome important limitations to make them more extensively applicable.

Among the most common mixed culture carbohydrates fermentation products are lactic, formic, acetic, propionic, butyric and valeric acids, ethanol, propanol, butanol, carbon dioxide and hydrogen (Bückel, 1999; Moat *et al.*, 2002). The prevalence of one or other products depends on several factors as the type of substrate, environmental conditions of the culture and ecological interactions within the mixed microbial population.

1.2. Energetics of anaerobic fermentations

Microorganisms need a source of energy to stay alive and to multiply. Most bacteria obtain energy from converting energy-rich to relatively energy-poorer chemical compounds in a series of catabolic reactions. Catabolism coupled to anabolism, biosynthetic reactions leading to new cells, constitute the metabolism. Fermentative and respirative metabolism can be distinguished (apart from photosynthetic conversions) as the mechanisms for energy release in bacteria. Fermentative metabolism occurs in absence of external electron acceptors (e.g. oxygen, nitrate, sulphate, etc) and the energy yield per unit of substrate converted is much lower compared with respirative metabolism. As a consequence, for growing or sustaining equal amounts of biomass, much larger amount of substrate must be converted in fermentative than in respirative metabolism. This means that larger amounts of product are formed per unit of biomass. This high product yields make microbial fermentations attractive processes for production of chemicals (Jöbses, 1986).

Organisms with anaerobic fermentative metabolism face two main problems when catabolising organic compounds (Madigan *et al.*, 2003): (i) conserving some of the energy released as ATP and (ii) disposing the electrons removed from the electron donor compound. The energy is typically conserved in fermentations by ATP synthesis via substrate level phosphorylation while the redox balance problem is solved by production and excretion of diverse fermentation products.

There is a wide variety of fermentations from diverse substrates leading to many fermentation products and biomass. Fermentations are typically classified in terms of their substrates or their fermentation products (Madigan *et al.*, 2003). Some substrates provide sufficient energy for generation of ATP but some others are unable to be coupled to ATP synthesis directly by substrate level phosphorylation. The catabolism of these substrates to support microbial growth is linked to ion pumps that establish proton or sodium gradients across the membrane and subsequent chemiosmotic ATP formation (Mitchell, 1979).

Anaerobic metabolism is not that different from aerobic in their mechanisms of energy generation. Both conduct conversions of substrate materials coupled to the synthesis of ATP, the universal cellular energy currency. The main difference between both metabolisms is the smaller redox span between electron donors and acceptors available in the anaerobic environments. This is responsible for the lower energy yields of anaerobes and their need to adopt diverse mechanisms to gain sufficient energy to drive growth and maintenance (Hamilton, 1988).

In anaerobic fermentations an organic substrate is oxidised in a series of reactions where the electron acceptor is a metabolic product of that substrate. The energy for ATP synthesis is provided by the difference in the redox potential between the substrate and the electron acceptor and ATP is normally generated by the mechanism of substrate level phosphorylation (SLP) (Moat *et al.*, 2002).

The accumulation of reduced fermentation products is needed to accomplish with redox balance. These reduced and only partially degraded products are consequently potential sources of energy, carbon and/or reducing power for other microorganisms.

Figure 1.1 shows in the upper part the number of donable electrons per carbon mol when the species is fully oxidised to carbon dioxide. In the lower part Figure 1.1 presents the energy content per carbon mol (or per mol) of several fermentation products. The energy content for glucose is defined as zero in order to illustrate the energy jumps to higher or lower energy content products, providing a view of the fate of energy. Thus, when glucose is fermented completely to a combination of products, to close the redox balance, part of the energy is conserved in more reduced higher energy content products, which can be of interest.

When studying fermentative mechanisms it is not possible to separate energetics and ATP production from carbon fluxes and redox balance. In fermentation with no external electron acceptor, the maintenance of the redox balance (reduction of the electron carriers coupled to their reoxidation) is a determinant influence on the carbon flux pathways and therefore on the consequent energy yield.

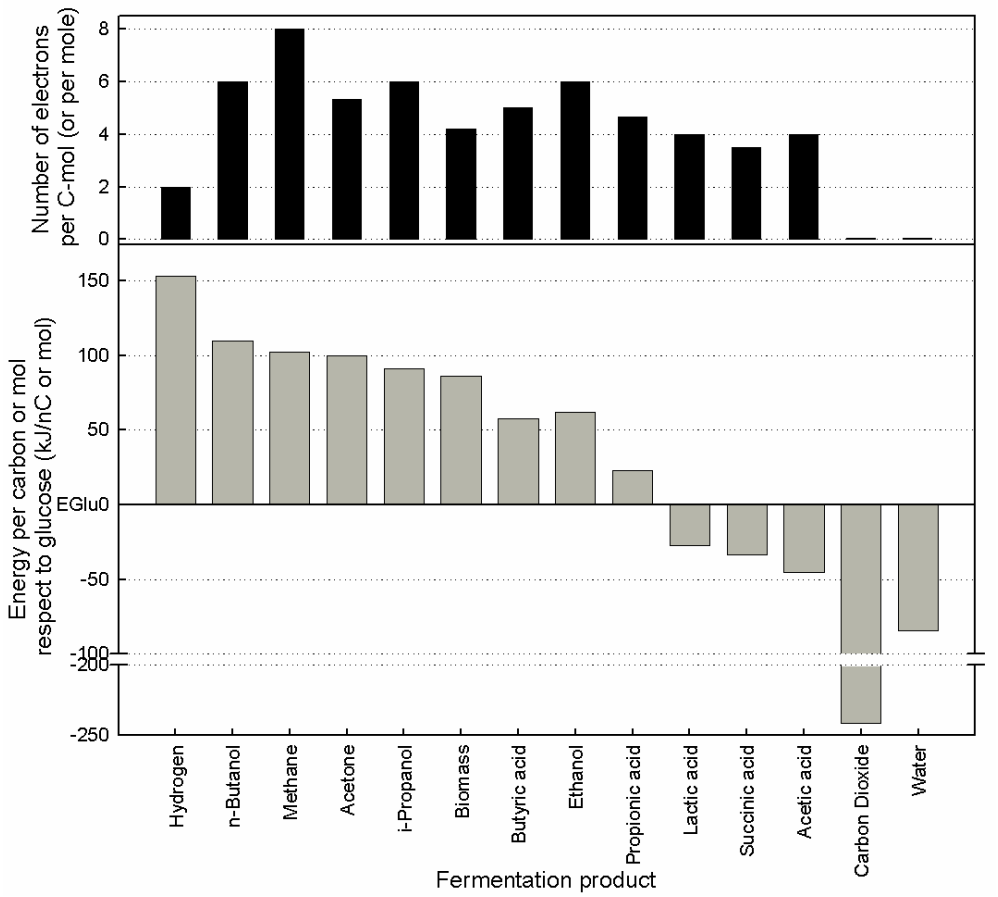

Figure 1.1. Number of donable electrons (top) and free energy content respect to glucose (bottom) per carbon atom (or per mole) of the most common fermentation products.

Another important point is the thermodynamic feasibility of the conversions. Figure 1.2 provides the standard (at pH 7) Gibbs free energy dissipated per electron in the oxidation semireactions of glucose and the most common fermentation products to water and carbon dioxide. It is shown how glucose is the most favourable to be oxidised and methane is the less favourable.

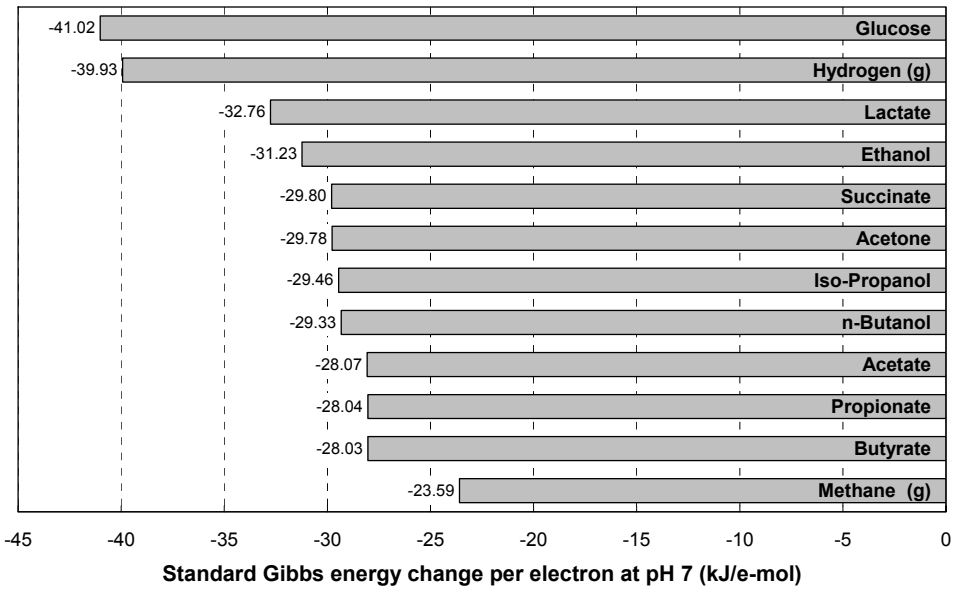

Figure 1.2. Standard (at pH 7) Gibbs energy dissipated per electron transferred in the oxidation semireactions of the species to carbon dioxide and water.

Very exhaustive studies on the energetics of bioprocesses and particularly anaerobic bacteria are available (Thauer *et al.*, 1977; Hanselmann, 1991; Heijnen and van Dijken, 1992; Westerhoff, 1982; Hellingwerf *et al.*, 1982). Energetics of anaerobic processes, as opposite to aerobic ones, has in general small driving forces, very close to equilibrium. This makes equilibrium assumptions in these processes closer to reality than when applied to other processes clearly far from equilibrium. Thermodynamic equilibrium provides an additional source of functional relations for modelling these anaerobic systems.

1.2.1. Main fermentation pathways

Anaerobic fermentation yields different amounts of ATP through the different pathways by substrate level phosphorylation (SLP) and/or by generation of proton motive force (*pmf*). In this section the main fermentation pathways and their energetics are briefly presented. Most of these pathways can be conducted by more than one microbial species in a mixed culture environment.

Depending on the species, sugars as main substrates are converted to pyruvate either via (i) the Emden-Meyerhof-Parnas (EMP) pathway, yielding 1 ATP per pyruvate formed or via (ii) the Entner-Doudoroff pathway yielding 0.5 ATP per pyruvate formed. Pyruvate can be considered as the central metabolite for the most relevant fermentations.

A summary of the most important fermentation patterns is given below (Hamilton, 1988; Moat *et al.*, 2002; Madigan *et al.*, 2003; Bückel, 1999).

i) Ethanol fermentations

Ethanol is one of the most known fermentation products as it has one the longest history as biotechnological product. Ethanol appears mainly in yeasts but also in some bacteria from sugars fermentations and mainly through pyruvate decarboxylation. The yeast *Saccharomyces* and some bacterial species as *Sarcina*, *Erwinia* or *Zymomonas* produce ethanol through pyruvate decarboxylation.

Ethanol is also a common fermentation product in many other bacteria but the normal bacterial pathway does not involve pyruvate decarboxylation. Rather, acetyl-CoA is formed as an intermediate and then reduced to ethanol by two NADH mediated reactions involving acetaldehyde and alcohol dehydrogenases, according to:

$$\text{Acetyl-CoA} + \text{NADH} + \text{H}^+ \rightarrow \text{Acetaldehyde} + \text{CoASH} + \text{NAD}^+$$

$$\text{Acetaldehyde} + \text{NADH} + \text{H}^+ \rightarrow \text{Ethanol} + \text{NAD}^+$$

With this mechanism, redox balance is only attained if ethanol conversion is accompanied by production of other more oxidised product, as it happens in mixed fermentations.

ii) Lactate fermentations

Lactic acid bacteria produce lactic acid by fermentation and when lactate is the sole product, as with *Streptococci*, *Pediococci*, *Sporolactobacilli* and most species of *Lactobacilli*, the mechanism is described as homofermentative. Glucose is converted to pyruvate via EMP pathway and then reduced to lactate, reoxidising the NADH formed and closing the redox balance. The energy yield is of 2 ATP per glucose.

$$\text{Glucose} + 2\cdot\text{ADP} + 2\cdot\text{P}_i \rightarrow 2\cdot\text{Lactate} + 2\cdot\text{ATP}$$

Heterofermentative metabolism, found in *Leuconostoc* and some *Lactobacilli*, ferments glucose to lactate, ethanol and carbon dioxide with a smaller energy yield of 1 ATP per glucose.

A third mechanism is the bifidum pathway found in *Bifidobacterium* where two glucose molecules yield 2 lactate and 3 acetate molecules. Energy yield is 2.5 ATP per glucose, higher than homo- and heterofermentative pathways.

iii) Butyrate and solvent fermentations

The obligate fermentative *Clostridia* produce acetate, butyrate and solvents as acetone and butanol as well as hydrogen. Pyruvate-ferredoxin-oxidoreductase plays a central role in this fermentation pattern for acetyl-CoA formation from pyruvate according to:

$$\text{Pyruvate} + \text{CoASH} + \text{Fd}_{ox} \rightarrow \text{Acetyl-CoA} + \text{CO}_2 + \text{Fd}_{red}$$

Ferredoxin is a low-redox-potential electron carrier and the reduced ferredoxin is close to equilibrium with molecular hydrogen ($\text{Fd}_{red} + 2\text{H}^+ \Leftrightarrow \text{Fd}_{ox} + \text{H}_2$). The spectrum of remaining fermentation products depends on the fate of acetyl-CoA: (i) It can be reduced to ethanol or produce acetate with 1 ATP generation; (ii) Alternatively two molecules of acetyl-CoA can condense into acetoacetyl-CoA and from there, butyrate or acetone and butanol as final fermentation products. Alcohols are highly reduced and acetate and butyrate are associated with additional ATP synthesis. By variating the fluxes through the different metabolic routes, the redox balance can be maintained while showing variable products and energy yields.

iv) Mixed acid fermentations

Enterobacteria present also complex fermentation patterns as *Clostridia*, involving many pathways and end products. Acetyl-CoA, hydrogen and CO_2 are again key products of pyruvate catabolism. Acetyl-CoA may be either reduced to ethanol or transformed to acetate with ATP production and formate is produced and converted to CO_2 and hydrogen. Lactate is also an important product formed directly from pyruvate according to:

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \rightarrow \text{Lactate} + \text{NAD}^+$$

These products as well as succinate are characteristic of the genera *Escherichia*, *Salmonella* and *Shigella*. Others as *Enterobacter*, *Serratia* and *Erwinia* produce less acids and more ethanol, CO_2 and in particular acetoin and 2,3-butanediol.

v) Propionate fermentation

Propionate is produced from pyruvate and lactate via two different pathways by a variety of organisms. Propionibacteria and most other propionate producers use a pathway involving transcarboxylation of pyruvate to oxaloacetate and propionate formation proceeds via the intermediate succinate. Succinate comes from the reduction of fumarate coupled to the generation of primary metabolic energy as proton motive force, which can be subsequently converted into ATP.

The other pathway, found in *Clostridium propionicum* and *Megasphera elsdenii* proceeds via the intermediate acryloyl-CoA. The fermentation balance from lactate is identical to that obtained with the succinate pathway but through a less energized route.

v) Acetate fermentation

Acetate is a major product in several fermentation patterns and it has a particular importance because its production is associated with increased ATP yield by SLP. However since it is an oxidised product, its production must be accompanied by other more reduced products.

Production and consumption of acetate and hydrogen by different species of microorganisms is an important point in the series of interacting metabolic activities in mixed cultures. This chain leads from carbohydrates, proteins and lipid polymers to the final products depending on the environmental conditions.

The so-called acetogenic bacteria, whose knowledge increased recently, can be divided in two subgroups, homoacetogens -fermenting to acetate as sole fermentation product- and hydrogen producing acetogens –transforming alcohols and organic acids into acetate and hydrogen. The acetogens, coupled with the other fermentative bacteria, ensure that all substrate materials can ultimately be converted into acetate and hydrogen.

vi) Methanogenesis

The biological production of methane is carried out by the strictly anaerobes *Archaea*. The production of methane occurs via a series of reactions of enormous complexity. Methane is produced mainly from reduction of carbon dioxide or from methylated compounds as acetate or methanol.

The reduction of CO_2 is generally driven by molecular hydrogen but other compounds can supply electrons as well. The reduction of CO_2 to methane yields energy enough for ATP synthesis by generation of proton motive force in one of the reduction steps.

Methane can be formed as well from a variety of methylated compounds. When acetate is the substrate for methanogenesis it is first activated to acetyl-CoA and the methyl group is transferred and goes through the terminal step of methanogenesis mediated by CoM. Methanogenesis from methyl compounds is linked to a proton pump (heterodisulfide reductase) for metabolic energy generation.

1.2.2. Membrane transport processes

The energy required by living cells to drive energy-demanding processes, for maintenance and growth, is called metabolic energy. In addition to ATP as the most important metabolic energy carrier, electrochemical energy stored in ion gradients (mainly protons or sodium-ions) is available as metabolic energy. Microbial cells, in absence of external electron acceptors, obtain metabolic energy by substrate level phosphorylation and by chemiosmotic free-energy-conserving processes (Mitchell, 1968; Konings *et al.*, 1995).

In chemiosmotic free energy conservation, chemical, light, or redox energy is converted into electrochemical energy by coupling the chemical reactions to ion translocations across the membrane. In most bacteria, protons are translocated but sodium ions are also the major coupling ions in a number of bacteria in energy-transducing processes.

The chemiosmotic hypothesis postulates that proton translocations by primary proton pumps generate electrochemical proton gradients or proton motive force across the cytoplasmic membrane. This gradient consist of two components: (i) The first component is electrical given by an electrical potential difference ($\Delta\psi = \psi_{in} - \psi_{out}$ in V), being the cell usually negatively charged inside respect to outside; (ii) The other component is chemical,

given by the pH difference (ΔpH) between inside and outside the cell. The value of the *pmf* in V can be calculated according to Eq. 1.1 where R, T and F have their usual meanings.

$$pmf = \Delta\psi - \ln 10 \cdot \frac{R \cdot T}{F} \cdot \Delta pH \qquad \text{Eq. 1.1}$$

By a net outwards translocation of protons, which can be mediated by several energy coupled mechanisms, a ΔpH inside alkaline can be generated. These mechanisms can involve primary or secondary transport of solutes.

The primary transport systems, as electron transfer systems or membrane-bound ATPases, generate the electrochemical ion gradients that exert a force called ion motive force. These membrane-bound electron transfer systems are the main responsible for proton motive force (*pmf*) generation in bacteria that use redox energy. The membrane-bounded ATPase that acts as a reversible proton pump, can generate or consume ATP when protons are imported or exported respectively.

Recently primary metabolic energy generation appeared not to be the only process generating *pmf* (Konings *et al.*, 1994) and in fermentative bacteria, metabolic energy can be generated by secondary transport processes. These secondary transport systems convert the chemical energy of a solute gradient into electrochemical energy of proton (or sodium ion) gradients (Poolman and Konings, 1993). Certain products as lactate can be transported in symport with protons (Konings, 1985). The number of protons symported depends on the pH gradient (Konings and Booth, 1981) and for values higher than one, metabolic energy is generated as *pmf*. Figure 1.3 shows primary and secondary transport mechanisms and the corresponding driving forces considering Eq. 1.1.

Fermentative microorganisms, in absence of external electron acceptors, are thus able to harvest the energy from the metabolic conversions, partially by means of SLP but also by ion motive energy associated to solute transports. Figure 1.4 presents an overview of the metabolic energy mechanisms in the cells.

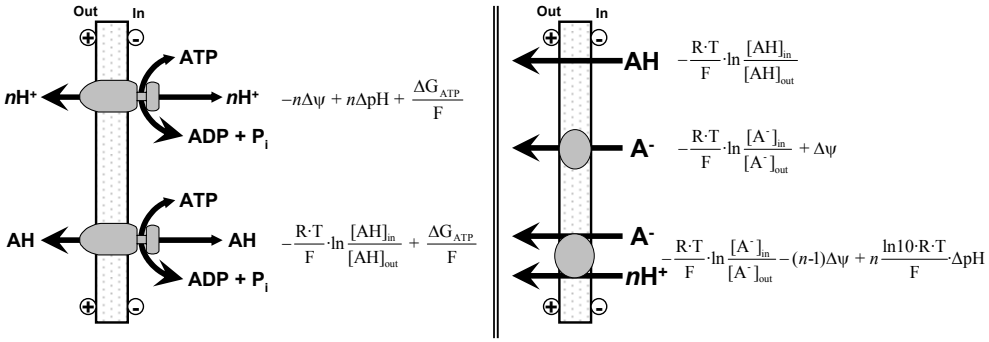

Figure 1.3. Primary (left) and secondary (right) transport mechanisms involved in weak organic acid export and their corresponding driving forces.

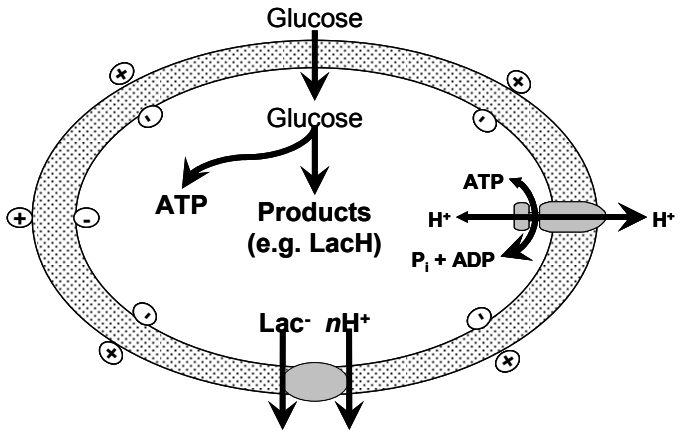

Figure 1.4. Overview of the metabolic energy in the cell. ATP synthesis by SLP; generation of secondary metabolic energy by solute export (e.g. lactate) in symport with a variable number of protons *n*; generation of primary metabolic energy by membrane bound ATPase.

1.3. Mathematical modelling

Real systems can be abstractly represented by means of ideas about their components and functional relations leading to conceptual models. There are many types of models: visual, linguistic, mental, physical, mathematical, etc. Depending on the purpose, one or other type will be the most adequate to be used. Some models are more appropriate for illustrative of qualitative descriptions while others allow quantitative predictions of the system under study (Andrews, 1993). The mathematical formulation of these ideas leads to a mathematical model of the system that can be used for quantitative description of its behaviour under certain external conditions.

In essence a mathematical model can be conceived as a machine transforming inputs (u) into outputs (y) through established relations. The characteristics of these relations between inputs and outputs define the basic structure type of model, which can be either a input-output of a state-space description (Dochain and Vanrolleghem, 2001). The outputs are variables of interest for the model user while the inputs are the variables manipulated and/or perturbations that affect the outputs.

An input-output model consists only of the transfer function converting inputs (u) into outputs (y). On the other hand a state-space model introduces the so-called state variables (x) as intermediate between inputs and outputs. The state of the system at a certain instant of time is defined by the state variables (x) obtained from their past values and the inputs (u) through the so-called state-transition equation. The outputs (y) are generated from the state variables (x) through the so-called observation equation. General expressions for both equations are presented in Eq. 1.2 and Eq. 1.3 where f and h are the state-transition and the observation equations respectively and θ is the parameter set of the model (Dochain and Vanrolleghem, 2001).

$$\frac{dx}{dt} = f(x,u,t,\theta)\,; \quad x(t=0) = x_0 \qquad \text{Eq. 1.2}$$

$$y = h(x,u,t,\theta) \qquad \text{Eq. 1.3}$$

The constituents of the mathematical expressions relating inputs to outputs can only be variables, constants or parameters irrespective of the model structure. (i) Variables are the inputs, outputs and states; (ii) constants are those constituents that never change their value for any model applications (e.g. physical or geometric constants); and (iii) parameters are those constituents whose values change depending on the circumstances of application and therefore their values must be determined for each particular model application (e.g. kinetic or stoichiometric parameters).

Models can be classified attending to different characteristics, a first option is to classify them as mechanistic or phenomenological models. (i) Mechanistic (also physical or white-box) models have a structure based on physical, chemical or biological laws. The sole source of information required for developing these models is the prior knowledge and they result from a deductive modelling strategy. (ii) Phenomenological (also empiric or black-box) models have a structure based on empirism rather than on natural laws. The source of information is experimental and they result from an inductive modelling strategy (Juditsky *et al.*, 1995; Sjoberg *et al.*, 1995).

Grey-box or semiphysical models are those resulting of the combination of mechanistic and phenomenological approaches (Tulleken, 1992; Thompson and Kramer, 1994). These type of models present the advantage of better identifiability properties together with interpretability of their parameters (Carstensen *et al.*, 1996).

Another issue for characterising mathematical models is their level of complexity in terms of the number of constituent equations and parameters (Dochain and Vanrolleghem, 2001). It is not clear when a model should be considered simple or complex but in general, phenomenological models are formulated with less complexity than mechanistic ones.

The level of complexity/simplicity of a model can be defined with the attributes aggregated or segregated (Jeppsson and Olsson, 1993). A model is considered lumped or aggregated compared to a reference model when some model variables and equations are united in a simplified form. Lumping of variables results inevitably in model errors, thus a proper compromise between complexity and accuracy must the achieved, always in view of the model objectives.

1.3.1. The model building process

The process of building a mathematical model consists of several steps from its initial conception to its final application (see Figure 1.5). The model building process should start with a clear formulation of the objectives of the model. This is often forgotten and is especially critic when the model developer and the supplier are not the same person. A model objective properly defined should be present in all the model building steps. A prior knowledge is necessary and collected from experts and literature. Also, experiments may be conducted as well to obtain some basic information about the system. It is necessary then to define the conditions under which the model will work; this frame definition aims at choosing the most adequate type of model for the given system (Dochain and Vanrolleghem, 2001).

At this point candidate model structures are created for the system under study involving two steps. First, a synthesis part where all the hypotheses about the mechanisms governing the system must be assembled in an often creative procedure. It must be demonstrated however that a candidate model created at this stage is a good approximation of reality. A second analytic part consists of evaluating the model against experimental data and can be also called system identification or model calibration.

System identification must be considered not only simply a calibration process but an integral part of the process of developing scientific theories about the behaviour of a system, leading to acquisition of knowledge and generation of new hypotheses (Beck, 1989). Thus, the model building process is an iterative cycle where experiments evidence the areas where the model is deficient and must be improved eventually with new hypotheses. In most physical and chemical systems there is high quality *a priori* knowledge and the iteration cycle deals mainly with parameter estimation and solving minor uncertainties in the structure. This does not apply in general for biological systems whose inherent characteristics of uncertainty, non linearity and variability make necessary structural modifications through the iterative modelling cycle (Vansteekiste and Spriet, 1982). Structure characterisation methods become important when dealing with biological systems and the number of modelling iterations can increase substantially (Dochain and Vanrolleghem, 2001).

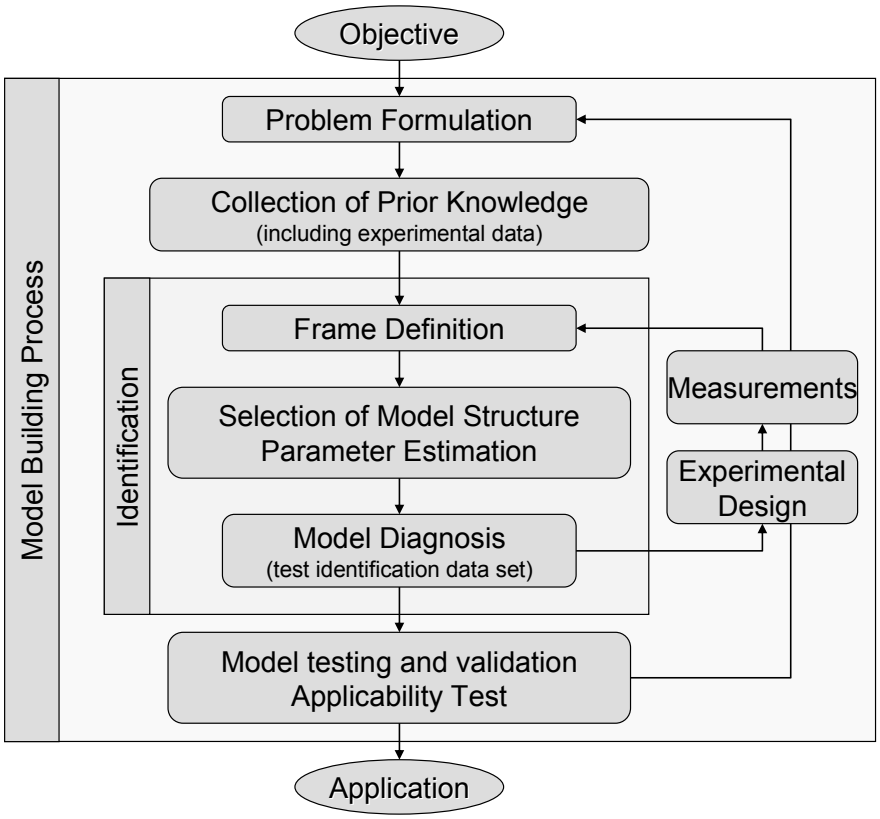

Figure 1.5. Model building process (adapted from Dochain and Vanrolleghem, 2001).

The model structure selection aims at selecting a unique model structure with the best quality (Spriet, 1985). Most model structure selection criteria require estimation of the parameter values, but methods requiring only basic data analysis are also available (Vanrolleghem and Van Daele, 1994).

Parameter estimation for a given model structure is typically based on the optimisation of a criterion assessing the quality of fit to experimental data and therefore the success depends strongly on the quality of the experimental data available. Identifiability studies bring light to the question whether for a set of data, unique values for the parameters can be obtained. A structural identifiability study evaluates theoretically whether the

parameters can be given unique values or not (Norton, 1986; Dochain *et al.*, 1995) while a practical identifiability study evaluates numerically whether the available data contain sufficient information to provide unique values for the parameters (Vanrolleghem *et al.*, 1995). A solution for practical identifiability problems can be obtained from optimal experiment design techniques to calculate, using the model, the experimental conditions able to generate data with maximum information for parameter estimation. On the other hand, model reduction techniques can lead to less data-demanding models and better identifiability properties (Dochain and Vanrolleghem, 2001). Once the model parameters are identified their values must be checked for violations of the assumptions made in the frame definition or non-sense parameter values.

The model building process culminates with the model validation step that consists of confronting the identified model with new sets of data but under conditions within the scope for which the model was originally defined. Successful validation results lead to a model ready for application.

1.4. Modelling of anaerobic digestion

The need for modelling microbial growth and product formation comes from the wish of understanding and describing these processes to ultimately control them. Mathematical models are however simplifications of reality and it is therefore possible to establish different mathematical models for the same process, it will depend on the objectives of the model and the available measurements (Bailey, 1998).

Anaerobic digestion converts organic matter into a gaseous mixture composed mainly of methane and carbon dioxide by means of the action of a bacterial community that carries out several fermentative conversions. The dominant species in anaerobic digesters are bacteria, less amounts of protozoa, yeasts and fungi have been also reported (Toerien and Hattingh, 1969). Anaerobic digestion is widely used for sludge stabilisation as well as for treatment of industrial wastewaters, organic fractions of municipal solid waste or agricultural wastes. During anaerobic digestion small amounts of sludge are produced and little nutrients and energy are required. Moreover high energy content methane is produced and can be used as energy source.

Anaerobic digestion modelling started on demand of efficient operation of anaerobic systems in the early 70's. Initially very simple models were developed because of the limited knowledge of the complexity of anaerobic digestion. Much more complex models have been developed during the last years due to the advances in the knowledge of the process and factors playing key roles in its performance and the immense progress in computing capacity. Interesting reviews about the efforts in modelling anaerobic digestion are available in the literature (Lyberatos and Skiadas, 1999; Gavala *et al.*, 2003; Pavlostathis and Giraldogomez, 1991)

The first modelling approaches focused on describing the limiting step of the process since anaerobic digestion is a multistep process and, as usually happens, these processes have one step that controls the global rate (Hill and Barth, 1977). The limiting step can however vary under different operating conditions (Speece, 1983). Some authors considered methanogenesis as the limiting step (Andrews, 1970), others the conversion of fatty acids into biogas or the hydrolysis of suspended solids (Eastman and Ferguson, 1981). Some models based on the limiting step were developed, incorporating first only acetoclastic methanogens, then acidogens and hydrolytic steps (Graef and Andrews, 1974; Hill and Barth, 1977; Kleinstreuer and Poweigha, 1982; Moletta *et al.*, 1986; Smith *et al.*, 1988). These first models based on the limiting step were simple and easy to use but were unable to describe well the process performance, especially under transient conditions.

A second generation of models considered the concentration of volatile fatty acids as the key parameter, incorporating separately acidogenesis and acetogenesis (Hill, 1982) and considering hydrolysis as well (Bryers, 1985).

The hydrogen partial pressure appeared as a key regulatory parameter influencing the redox potential in the liquid phase and more bacterial groups, with differentiated acetoclastic and hydrogenotroph methanogens, were included in several models (Mosey, 1983; Costello *et al.*, 1991; Ruzicka, 1996; Pullammanappallil *et al.*, 1991). The redox potential (as $NADH/NAD^+$ ratio) is function of the hydrogen partial pressure and determines the VFA production in this family of models.

Further microbiological studies and new concepts (McCarty and Mosey, 1991) led to the most recent generation of models (Angelidaki *et al.*, 1993; Siegrist *et al.*, 1993; Vavilin *et al.*, 1994; Vavilin *et al.*, 1995; Kalyuzhnyi and Davlyatshina, 1997; Kalyuzhnyi, 1997; Kalyuzhnyi *et al.*, 1998; von Munch *et al.*, 1999; Angelidaki *et al.*, 1999; Batstone *et al.*, 2000; Bernard *et al.*, 2001; Tartakovsky *et al.*, 2002). These models incorporate much knowledge about the anaerobic digestion process by including more processes and species, more complex kinetics with inhibition and considering different substrates.

As a response to the need of a generic model for anaerobic digestion, a very important effort was made by the IWA Task Group for Mathematical Modelling of Anaerobic Digestion Processes that presented the generic Anaerobic Digestion Model No.1 (ADM1) (Batstone *et al.*, 2002) providing, among other advantages, a common basis for further model development and validation studies with comparable results. The ADM1 is a general purpose model of the anaerobic digestion including the most important processes. Its main application is for dynamic systems, plant design, operation and optimisation purposes as well as training purposes (Bernard, 2005).

The ADM1 can predict the dynamics of 24 species and includes 19 bioconversion processes and this led to a model with a large number of parameters. Given its general purpose the ADM1 neglects some processes and species of more specific applications to avoid extreme complexity. Still, the high number of parameters and identifiability difficulties are the major drawbacks for ADM1 application as well as some structural weak points that have been recently addressed (Kleerebezem and van Loosdrecht, 2004).

Many model developments and applications using ADM1 appeared in the recent years (Batstone *et al.*, 2005b). Some authors applied the model to stirred tank systems and others presented distributed parameter applications (Batstone *et al.*, 2004a; Picioreanu *et al.*, 2005; Batstone *et al.*, 2005a). Extensions have been developed to incorporate processes absent in the original model and applications to particular types of wastewater have been also published (Fedorovich *et al.*, 2003; Batstone and Keller, 2003; Batstone *et al.*, 2004b; Batstone *et al.*, 2003; Ruiz *et al.*, 2004; Elmitwalli *et al.*, 2003). Efforts has been done in the recent years also in simplifications of the model (Siegrist *et al.*, 2002; Copp *et al.*, 2004), providing parameters values for use with the ADM1 (Batstone *et al.*,

2003; Siegrist *et al.*, 2002; Batstone *et al.*, 2004a; Blumensaat and Keller, 2005; Parker, 2005). Methodologies for easy application of the ADM1 (Zaher *et al.*, 2004; Kleerebezem and van Loosdrecht, 2005) or for structural simplifications of the model under certain conditions (Bernard *et al.*, 2005) appeared as well.

The framework provided by the ADM1 will be useful especially for design, dynamical and control purposes but, due to its fixed stoichiometry approach, its applicability would require important structural modifications in certain processes. Implications of structural changes in some processes of the ADM1 towards a variable stoichiometry structure have been recently analysed (Rodríguez *et al.*, 2005).

Future perspectives in modelling anaerobic bioprocesses lay on the production of chemicals from anaerobic fermentation. The way the modelling of these processes is tackled and its scope for general or particular purposes, is crucial when deciding the modelling technique to apply and the level of complexity in terms of compounds and processes as well as the relations among them to be considered.

1.5. Metabolic modelling

When tackling optimisation of fermentation processes two -not mutually exclusive- directions can be taken. They can be focused on the maximisation of either the production rates or the product yield. The type of process, its performance, economic aspects, downstream processing and more issues must be considered to distribute efforts in one or other direction (Vanrolleghem and Heijnen, 1998).

Metabolic models incorporate biochemical information of the microorganism conducting a certain conversion. Metabolic information about the microorganism under study has to be collected and afterwards candidate models can be set up. In the recent years extensive work has been done in the metabolic modelling field making use of the new molecular tools and computational power available (Stephanopoulos, 1999; Stephanopoulos *et al.*, 1998). Most of the metabolic models focus on improvement of pure culture bioprocesses where valuable products or biomass are obtained (van Gulik and Heijnen, 1995).

Metabolic conversions are known to be a combination of a huge number of reactions that form a reaction network. Metabolic flux analysis (MFA) has been widely used for the quantification of the intracellular fluxes in the metabolism of microbial cells. Mass balances over the intracellular metabolites are used to calculate the fluxes through the different branches of the network (Gombert and Nielsen, 2000). The fluxes can be calculated by combining measurements with linear algebra or linear optimisation (Varma and Palsson, 1994). The use of labelled substrates combined with the measurement of the labelling state of intracellular metabolites has been used more recently to estimate the fluxes (van Winden, 2002).

To obtain the stoichiometric model of a microbial metabolism, a stoichiometric matrix describing appropriately the metabolism is initially defined, based on the biochemical information available. The matrix typically contains the species in rows and the stoichiometry of the conversions in columns. Multiplication of the stoichiometric matrix (S) by the vector of fluxes or reaction rates of the conversion processes (v) provides the vector of net generation rates of all the species (R) (see Eq. 1.4). For steady state situations this net generation must be zero for the conserved moieties and therefore the mass balance equation is fulfilled by any vector (v) living in the null space of the matrix (S).

$$\mathrm{S}_{(s \times p)} \cdot \mathrm{v}_{(p \times 1)} = \mathrm{R}_{(s \times 1)} \qquad \text{Eq. 1.4}$$

Depending on the objectives, diverse algebraic manipulations of the matrix can be carried out. Imposition of constraints as measured fluxes can lead to a full determination of the system that becomes soluble by linear algebra; labelling techniques can provide further constraints to the system. When the system cannot be constrained leading to a determined system, linear optimisation techniques can be applied to a suitable objective function. Other manipulations like those leading to the calculation of the so-called elementary flux modes can provide more insight into the metabolism studied (Gombert and Nielsen, 2000).

Metabolic approaches can be not sufficient to achieve comprehensive understanding of anaerobic bioenergetics in terms of cellular mechanisms or wide ecological implications. In anaerobic conditions the many interspecies relations are integral to the energetics and metabolism of the individual species and they may be required for the individual viability.

1.6. Ecological issues in anaerobic ecosystems

Individual species of anaerobic microorganisms have a relatively restricted metabolic potential and therefore there are key interdependencies among species. Any analysis of anaerobic microbial ecosystems must consider always the interactions between individual components of the community (Hamilton, 1988).

In a mixed culture, where many microbial species are present, most of the fermentative conversions explained above are possible. The fermentation patterns observed will be a result of the combined effect and interactions among the microbial species present (Rittmann and McCarty, 2001; Madigan *et al.*, 2003).

From an ecological perspective not only the species present, their relative quantity and also their spatial arrangement (e.g. formation of aggregates or biofilms) affect the global performance. The different microbial species interact among them and the individuals most fit to survive in the given environment are selected.

The role of the exchange of materials between cells is a very important issue. Fermentation products for certain species are substrates for others, creating food chains in the microbial community. In some cases the survival of certain species is conditioned to the depletion of its metabolic products by other species and this second depends on the first to obtain substrates. Not only nutrient material is exchanged, genetic material or growth factors and in some cases chemical signals to trigger certain physiological responses are also exchanged among microbial cells.

Microbial ecosystems are also capable to respond very dynamically to changes in their environment and particularly to stress. Adaptation to stress as changes in temperature, pH, salinity, toxics or starvation, occurs and leads the community to maintain its function under the new conditions or to eliminate the stress by changing the medium, this illustrating the interdependence between life and medium (Lovelock, 2003). In some cases the community structure changes drastically but it can also remain intact even with greatly altered function. Many important principles about microbial ecology, diversity and stability are still unclear and under active investigation (Moore, 2005).

All these interactions play important roles in the performance of microbial communities and add complexity to these systems. However, as for all processes in nature, their performance is subject to the conservation laws of mass, redox and energy as well as to the thermodynamics. Complete application of these principles is very useful to achieve a better understanding of these systems and to apply them for technological purposes.

1.7. Outline of this thesis

This thesis intends to contribute to the field of modelling anaerobic fermentation processes by assessing the problem from different perspectives including a rather new bioenergetic approach.

The work described in this thesis deals with the development and application of models for anaerobic fermentations. Motivations as well as some background have been presented in Chapter 1 where modelling challenges on view of the current environmental and energetic situation can be identified.

The increasing interest in processes for production of chemicals from wastes using mixed cultures makes their modelling of enormous importance if an appropriate control of these processes is desired. In Chapter 2 a new modelling approach for prediction of product formation in anaerobic mixed culture fermentations is presented and an initial and simple model is developed. The model tries to predict the effect of the environmental conditions of the mixed culture on the product spectrum obtained in steady state, using an optimum biomass growth criterion, limited by energy availability as ATP on a completely variable stoichiometry basis.

Since the initial model obtained presents an important number of limitations and could not predict some patterns observed experimentally, alternatives for improvement under the same modelling approach are investigated. Chapter 3 analyses the objectives and limitations and presents a discussion of the most relevant issues to be considered and incorporated for further model developments as well as partial results of the exploration of some of the alternatives.

In Chapter 4 the Anaerobic Digestion Model No.1 (ADM1) is introduced and applied to simulation of the anaerobic treatment of ethanolic wastewater. Through a structural modification, ethanol degradation is incorporated on a fixed stoichiometry basis following the ADM1 structure and the new parameters are obtained from experimental on-line measurements under overload conditions. This approach provides acceptable fitting results useful for limited control purposes. The important limitations of these kind of model calibration approaches, using fixed stoichiometry and observed kinetics, are discussed.

The implications of integrating the variable stoichiometry approach, presented in Chapter 2 and Chapter 3, with the ADM1 are presented in Chapter 5. The results obtained gave rise more to interesting new questions than to answers, about the most adequate methodology to tackle the modelling of anaerobic fermentative and methanogenic systems, on a variable stoichiometry basis.

Finally Chapter 6 presents and applies a statistical tool to asses the complexity required when modelling a certain anaerobic process, to reproduce the dynamics under a certain experimental range. Principal component analysis (PCA) is applied to several sets of experimental data from overload situation in anaerobic reactors with different types of wastewater. The PCA tool permits to assess the model complexity required to predict their variability but also serves as a tool for reduction of complex models. This work seeds a useful tool for the assessment of model complexity and for models simplification. The PCA methodology can be applied to any modelling problem in the field and will reduce the common drawbacks derived from inadequate correlations between model scope and model complexity.

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Chapter 2

MODELING PRODUCT FORMATION IN ANAEROBIC MIXED CULTURE FERMENTATIONS

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Chapter 2

MODELLING PRODUCT FORMATION IN ANAEROBIC MIXED CULTURE FERMENTATIONS

Contents

Abstract

A new approach for modelling product formation in mixed culture anaerobic fermentations is presented in this chapter. A model was developed to illustrate this approach that considers the mixed culture as a virtual microorganism, whose metabolic network consists of the most common pathways for glucose fermentation. The performance of anaerobic systems, very close to thermodynamic equilibrium, enabled the application of energetic limitation criteria to the system. The product formation is predicted by maximising the biomass growth in the metabolic network constrained by the thermodynamic feasibility under the environmental conditions provided. Preliminary results of the model reproduced the main trends observed in the literature for acetate and butyrate as main catabolic products.

2.0. Summary

The anaerobic conversion of organic matter to fermentation products is an important anaerobic biotechnological process. The prediction of the fermentation products is until now a complicated issue for mixed cultures. A modelling approach is presented here as an effort to develop a methodology for modelling fermentative mixed culture systems. To illustrate this methodology a steady-state metabolic model is developed for prediction of product formation in mixed culture fermentations as a function of the environmental conditions. The model predicts product formation from glucose as a function of the hydrogen partial pressure (P_{H2}), reactor pH and substrate concentration. The model treats the mixed culture as a single virtual microorganism catalyzing the most common fermentative pathways, producing ethanol, acetate, propionate, butyrate, lactate, hydrogen, carbon dioxide and biomass. The product spectrum is obtained by maximising the biomass growth yield which is limited by catabolic energy production. The optimisation is constrained by mass balances and thermodynamics of the bioreactions involved. Energetic implications of concentration gradients across the cytoplasmic membrane are considered and transport processes are associated with metabolic energy exchange to model the pH effect. Preliminary results confirm qualitatively the anticipated behaviour of the system at variable pH and P_{H2} values. A shift from acetate to butyrate as main product when either P_{H2} increases and/or pH decreases is predicted as well as ethanol formation at lower pH values. Future work aims at extension of the model and structural validation with experimental data.

2.1. Introduction

Industrial fermentation processes are typically performed using pure cultures and aimed at the production of high value products. For bulk-chemical production the pure culture process seems less attractive due to the high costs, like those associated with control of the culture performance and with working under strictly sterile conditions in order to prevent contaminations. Important equipment investments are necessary for these processes at industrial scale as well and also pure culture fermentations require generally the use of pure and therefore more expensive substrates. The risk of contamination of the culture remains, since an unstable pure microbial culture has to be maintained.

Mixed cultures consist of a more stable mixed microbial population, as typically found in nature. The use of less pure substrates (even wastes or by-products) is possible with the subsequent costs implications. Mixed culture fermentations (MCF) did not find wide application at industrial scale because they present still important limitations. The products formed by MCF vary in amount and composition and the control of the optimum balance among the microorganisms is not straightforward and requires a better understanding of their behaviour (Hesseltine, 1991).

2.1.1. Production of chemicals from mixed culture fermentations

During extraction of many agricultural products, large amounts of residues are produced. The use of this organic matter by the biotechnological industry is limited due to the large diversity of organic compounds present in these residues. Production of energy carriers (Claassen *et al.*, 1999) or other valuable products by mixed culture fermentations would bring utility to those useless wastes or by-products and also enable interesting downstream integrations. MCF is a potentially interesting technology for validation of these streams and generation of specific products. Products that can potentially be obtained by MCF include mixtures of volatile fatty acids, alcohols or lactate that may serve as building blocks in other processes. Several interesting applications exist for MCF processes:

i) *Production of biodegradable polymers* such as 3-hydroxyalkanoic acids (PHAs), e.g. poly-3-hydroxybutyric acid (PHB), has been extensively investigated (Lee, 1996). PHA's can effectively be produced by mixed cultures of bacteria by imposing a strong selection pressure on the mixed culture (Reis *et al.*, 2003).

ii) *Biological hydrogen production* by MCF has a large research interest (Benemann, 1996) due to its potential application as energy carrier for several applications. The hydrogen production yield depends stoichiometrically on the range of fermentation products formed.

iii) *Solvent fermentations* for production of alcohols and acetone, butanol or propanol by clostridial cultures has been of much interest in past and recent years (Dürre, 1998) due to their potential application as sustainable additives to gasoline. To which extent mixed cultures can be applied to the production of specific solvents, remains largely unclear.

iv) The carbohydrate fermentation is furthermore a crucial step in the *anaerobic digestion process* for wastewater treatment and valorisation of solid waste streams. In anaerobic digestion processes of wastewater the initial fermentation of carbohydrates (acidogenesis) leads to a variety of products that are finally methanised by other microbial populations.

The interest of carbohydrate fermentations in the framework of all these applications motivates for modelling these processes from the perspective of control of the product formation.

2.1.2. Control of a mixed culture fermentation

The control of the product spectrum obtained during fermentation of carbohydrates by mixed cultures has been studied in the past decades and some models have been proposed. Most of these models described the fermentation of carbohydrates considering a fixed stoichiometry (Vavilin *et al.*, 1996; Kalyuzhnyi, 1997; von Munch *et al.*, 1999; Batstone *et al.*, 2002) mainly for integration in anaerobic digestion models. However, experimental investigation have repeatedly demonstrated the dependency of the products formed on the operational conditions (Zoetemeyer *et al.*, 1982a; Zoetemeyer *et al.*, 1982b; Horiuchi *et al.*, 2002). To describe the variable stoichiometry of product formation during MCF some models have been proposed that predict the effect of hydrogen on the product scheme based on thermodynamic considerations (Mosey, 1983; Costello *et al.*, 1991; Ruzicka, 1996). These authors however used thermodynamic or inhibitory hydrogen-dependent kinetic expressions for the fermentative reactions assuming a direct relation between thermodynamics and kinetics. These models do not incorporate biochemical information and do not predict pH effects since they focus mainly on the hydrogen effects.

Successful application of metabolic models to anaerobic metabolism, including the energetic effects of pH in the medium, can be found in the literature (Beun *et al.*, 2000; Kleerebezem and Stams, 2000). Here a modelling concept for prediction of the product formation in MCF is proposed. Metabolic network based assumptions are used leading to a simplified model of the MCF process. The model provides a mechanistic interpretation of the processes occurring and identification of the most relevant parameters is possible.

2.2. Model for anaerobic mixed culture fermentation

The objective of the model is to predict the formation of products in anaerobic MCF under steady state conditions. In this paper glucose is considered as the only carbon and energy source while the main anaerobic fermentation products are included.

The model proposed is based on the assumption that the fermentation products obtained will be those providing the maximum energy generation for growth processes to the mixed culture, under the given conditions in the medium. This assumption is based on the hypothesis that natural selection, as imposed by evolution, has selected microbial populations able to maximise their growth efficiency under any given environmental conditions (Westerhoff, 1982). This results in the dominance of the microbial community with the highest biomass production rate under the prevailing environmental conditions.

The model developed assumes a virtual organism capable of catalyzing the most typical pathways for glucose fermentation leading to the following range of products being considered: hydrogen, carbon dioxide, lactate, ethanol, butyrate, propionate, acetate and biomass. Besides the intracellular product formation pathways, transport of products across the cytoplasmic membrane is relevant. As opposed to a thermodynamic black box approach (Kohn and Boston, 2000), the grey box approach proposed here includes biological information and thereby reduces the number of degrees of freedom. The inclusion of common mechanisms and pathways, like energy coupling to transport processes enables mechanistic interpretation of the results obtained.

The virtual microorganism proposed here should be regarded as a representation of the different microbial strains involved in carbohydrate fermentation. Microbial diversity and dynamics of the process are neglected at this stage. The fact that bioreactions in anaerobic

processes typically run very close to thermodynamic equilibrium (Mcinerney and Beaty, 1988), can justify for the assumption that the virtual microorganism will behave similar to a microbial consortium under steady state conditions. Herewith it is assumed that metabolic efficiency dominates over phylogenetic diversity in the product formation and environment selects for the organism that is capable of catalyzing the thermodynamically most efficient set of reactions (Dollhopf *et al.*, 2001).

The core of the model developed consists of a bioreaction network where glucose and ammonium are the sole carbon and nitrogen sources respectively. The biomass growth is described as a general anabolic reaction fuelled by ATP. Finding the optimum fluxes vector through the biochemical network resulting in maximum biomass growth within the thermodynamic feasible region, provides the net formation rate of products and biomass for the given environmental conditions.

2.2.1. Bioreaction network: stoichiometry

A metabolic network of the whole mixed culture is built up just by inventorying the most common catabolic bioreactions known for glucose fermentation to the products considered (Bückel, 1999) and posterior lumping of the most important pathways occurring in a MCF of that carbohydrate. Figure 2.1 presents the bioreaction network proposed including the transport of substrates and products. Pyruvate is the central branching metabolite in all pathways except for the biomass production pathway that is defined directly from glucose. Only the NADH/NAD redox-couple is explicitly considered for electron transfer. The potential role of the FAD/$FADH_2$ electron carrier is identified and implicitly taken into account as will be outlined where required.

Based on the reaction inventory (Bückel, 1999), the following (lumped) reactions were considered (see Figure 2.1 for the general scheme of reaction network and Figure 2.2 for the algebraic stoichiometric representation):

v1: *Pyruvate formation* from glucose accompanied by ATP and NADH generation via the Embden-Meyerhoff pathway. Only the stoichiometry of this conversion is considered and with no kinetic description is established and consequently a pyruvate concentration needs to be assumed.

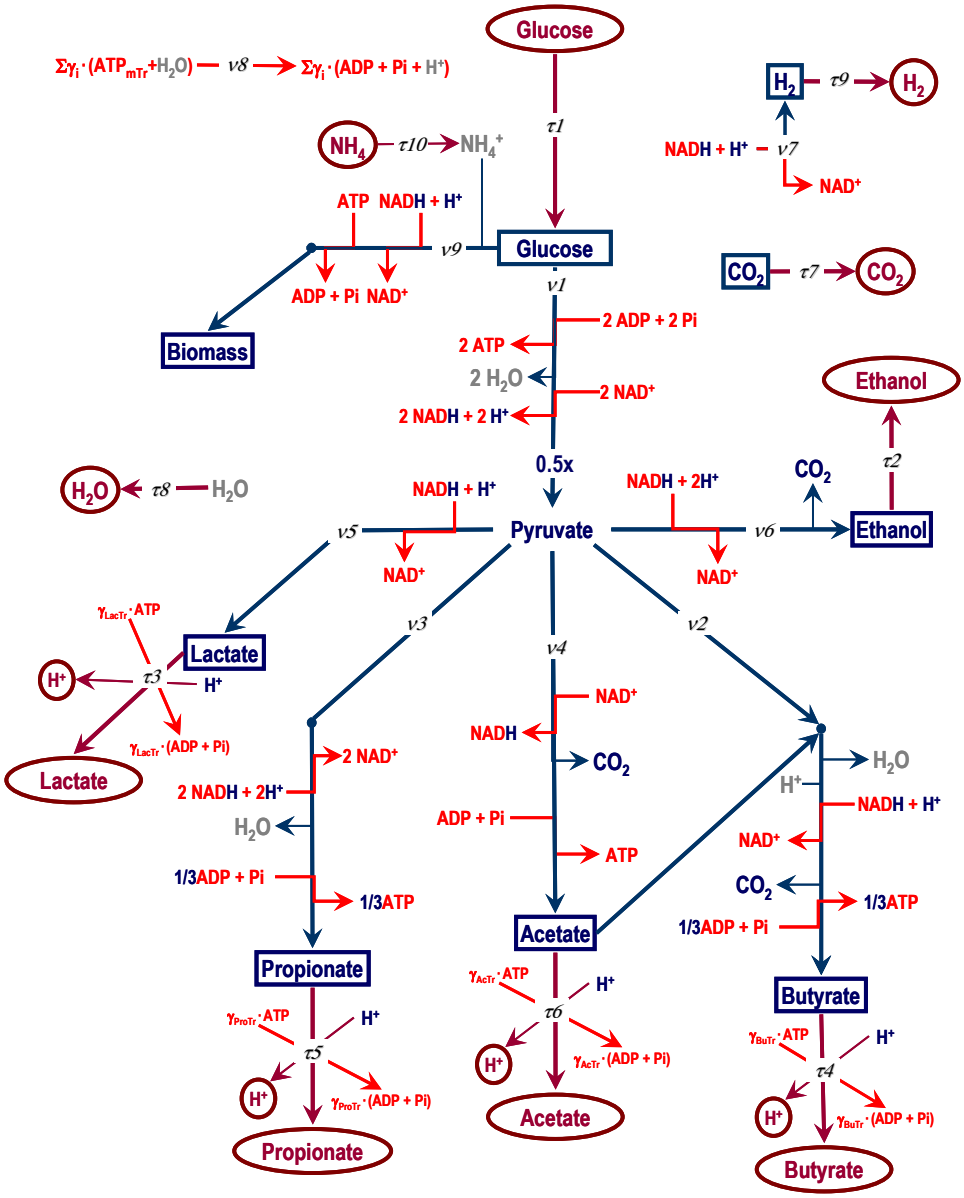

Figure 2.1. Bioreaction network proposed for the mixed culture fermentation of glucose.

v2: *Butyrate production* by reduction and decarboxylation of pyruvate, consuming one acetate. One of the bioreactions of the lumped pathway corresponds to the reduction of crotonyl-CoA to butyryl-CoA with $FADH_2$ as electron donor under formation of FAD. Subsequent reduction of FAD is assumed to be accompanied by NADH oxidation by translocation of one proton across the cytoplasmic membrane. Assuming a H^+/ATP yield of 3 this implies that crotonate reduction is accompanied by NAD^+ production and the formation of 1/3 ATP.

v3: *Propionate production* by reduction of pyruvate through the succinate-fumarate pathway lumped. Propionate formation is assumed to be accompanied by the production of 1/3 ATP during $FADH_2$ dependent fumarate reduction, analogue as in butyrate production.

v4: *Acetate production* by pyruvate oxidation and decarboxylation. One ATP is obtained by substrate level phosphorylation.

v5: *Lactate production* by reduction of pyruvate.

v6: *Ethanol production* by reduction and decarboxylation of pyruvate.

v7: *Hydrogen production* by oxidation of excess NADH. This reaction is assumed to proceed close to thermodynamic equilibrium. Herewith the hydrogen partial pressure has a direct impact on the oxidation state of the NADH/NAD couple and the thermodynamic state of the different product formation pathways (Mosey, 1983).

v8: *Hydrolysis of ATP* for maintenance and ATP driven transport processes that will be explained below.

v9: *Biomass growth* is set up as a lumped anabolic reaction (Heijnen, 2001) producing biomass from glucose and ammonium and using ATP as energy source, the detailed stoichiometry is shown in Figure 2.2.

The bioreaction network proposed (Figure 2.1) considers 18 intracellular species from which 12 can be exchanged with the environment and 6 are present only inside the cell, namely ADP, ATP, phosphate, pyruvate, NAD^+ and NADH. These are assumed not to be transported over the cell membrane and therefore considered as conserved moieties under steady state conditions.

Figure 2.2 presents the proposed network set-up in an algebraic form where the 18 first rows correspond to the intracellular species and the last 12 to the extracellular species. Columns correspond either to bioreactions or to transport fluxes, thus the first 9 columns are the bioreactions considered and the last 11 columns correspond to the transport of substrates and products defined for the network from Figure 2.1. Multiplication of the resultant 30x20 matrix by a vector of 20 fluxes (9 for bioreactions and 11 for transport) leads to a vector of net formation of all species.

		Bioreactions									Transport											Flux Vector	Net Flux
		v1	v2	v3	v4	v5	v6	v7	v8	v9	τ1	τ2	τ3	τ4	τ5	τ6	τ7	τ8	τ9	τ10	τ11		
Intracellular	Glucose	-1								-1	1												0
	Pyruvate	2	-1	-1	-1	-1	-1																0
	Ethanol						1					-1											0
	Lactate					1							-1										0
	Butyrate		1											-1									0
	Propionate			1											-1								0
	Acetate		-1		1											-1						v1	0
	Phosphate	-2	-0.33	-0.333	-1				1	9.8361												v2	0
	ADP	-2	-0.33	-0.333	-1				1	9.8361												v3	0
	ATP	2	0.333	0.3333	1				-1	-9.836												v4	0
	NAD+	-2	1	2	-1	1	1	1		-1.672												v5	0
	NADH	2	-1	-2	1	-1	-1	-1		1.6721												v6	0
	Carb. Dioxide		1		1		1			1.082							-1					v7	0
	Water	2	1.333	1.3333					-1	-8.459								-1				v8	0
	Hydrogen							1											-1			v9	0
	Amonium									-0.984										1		τ1	0
	Proton	2	-2.33	-2.333		-1	-2	-1	1	12.492			-1	-1	-1	-1				-1		τ2	0
	Biomass									4.918											-1	τ3	0
Extracellular	Glucose										-1											τ4	rGlu
	Ethanol											1										τ5	rEtOH
	Lactate												1									τ6	rLac
	Butyrate													1								τ7	rBu
	Propionate														1							τ8	rPro
	Acetate															1						τ9	rAc
	Carb. Dioxide																1					τ10	rCO2
	Water																	1				τ11	rH2O
	Hydrogen																		1				rH2
	Amonium																			-1			rNH3
	Proton												1	1	1	1				1			rH
	Biomass																				1		rX

Figure 2.2. Algebraic form of the metabolic network for MCF of glucose.

Application of mass balances to the intracellular species, whose net production must be zero in steady state, is performed by calculation of the null space of the 18 first rows of the stoichiometry matrix; a base of 6 vectors is thus obtained. All vectors of fluxes feasible in terms of mass balances must be a linear combination of the 6 base vectors of the null space obtained. The network has therefore 6 degrees of freedom after application of mass balances to the internal conserved moieties.

2.2.2. Thermodynamic constraints

Besides the mass balances, additional constraints to the biochemical reactions are imposed by the thermodynamics. The Gibbs free energy change of a certain bioreaction or transport process must be negative to be feasible with a positive flux. According to Eq. 2.1 the activities of substrates and products in a certain process affect the Gibbs free energy change of the reaction.

$$\Delta G = \Delta G^{o} + R \cdot T \cdot ln\left(\prod_{j} a_{j}^{\gamma_j} \right) \qquad \text{Eq. 2.1}$$

where a_j is the activity (typically concentration in diluted solutions) of the species S_j for a certain process/reaction with the stoichiometry

$$\gamma_1 \cdot S_1 + \gamma_2 \cdot S_2 + \gamma_3 \cdot S_3 + \ldots + \gamma_j \cdot S_j + \ldots = 0$$

This implies that the accumulation of fermentation products can lead to thermodynamic limitation of a certain bioreaction.

The concentrations and partial pressures of products in MCFs are dependent on liquid and gas handling in the reactor. The operational conditions, including the substrate (glucose) concentration degraded, will define the product spectrum obtained. The type of reactor (e.g. a chemostat), reactor pH (leading to higher or lower concentration of the undissociated forms of the acidic products) and partial pressures of gases (particularly hydrogen and CO_2 that play an important role) are the variables considered in this work.

2.2.3. Transport processes: bioenergetics

The number of ATP-molecules consumed and produced in the intracellular reactions *v1* to *v9* as shown in Figure 2.1 have all been identified from generalized metabolic schemes. Besides this direct metabolic energy, the bioenergetic implications of the transport of acidic species (*τ3* to *τ6*) are important in modelling the overall catabolic energy production, mainly when considering the effect of the medium pH. The transport of chemical species over the cellular membranes can lead to generation or consumption of proton motive energy depending on the environmental conditions (Konings *et al.*, 1995; van Maris *et al.*, 2004) given that the membrane potential can be converted into ATP equivalents (Mitchell, 1979).

Energy mediated transport is considered for the excretion of the undissociated acidic products. When the products must be transported against a concentration gradient active transport is considered with energy consumption as ATP. This implies that energy investment is required at high extracellular product concentrations.

The opposite situation happens at low extracellular concentrations when transporting an organic acid (acetate, propionate, butyrate or lactate) across the membrane down a concentration gradient and potential energy can be conserved by symport with a variable number of protons. This symport transport has been reported for lactate (Michels *et al.*, 1979; Konings *et al.*, 1995; Konings, 1985) and it was assumed that similar mechanisms apply for all the acid species in the model. Thus the energy available in the proton gradient generated can be conserved by a membrane bound ATPase (Konings *et al.*, 1995).

Full efficiency of the interconversions from any form of energy to ATP is assumed in the model. The energy change of transport of a certain species across the cellular membrane is thus due only to the concentration gradient (second term of the Eq. 2.1).

Besides the energy mediated transport of the acidic products, the non ionic forms of the products and substrates are assumed to diffuse freely over the cell membrane, without any energy change considered. Energy mediated transport is considered only for acidic products, since energy consumption for uptake of substrates (glucose and ammonium) will not affect the optimum catabolic product spectrum, but mainly the total energy and the biomass yield.

Figure 2.3 gives the scheme of the modelled transport processes; the performance at low and high extracellular pH values is shown. At lower extracellular pH, more energy is needed for transport of the undissociated acid product due to the higher concentration outside the cell (see Eq. 2.2). Furthermore, the free diffusion flux of the undissociated form of an acid leads to a futile cycle that consumes extra ATP for active transport of the metabolic production plus the product that diffused from the medium into the cell.

At higher extracellular pH, energy can be obtained from transport of the free form of the acid, if the metabolic production exceeds the free diffusion flux directed outwards.

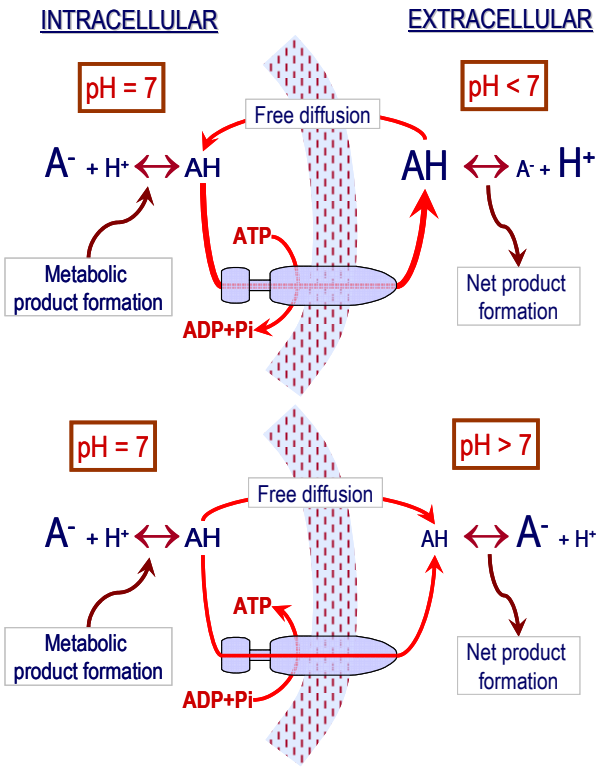

Figure 2.3. Model of transport of the acidic species through the cell membrane.

The energy required for active transport of an acid molecule *AH* through the membrane ($\Delta G_{Tr,AH}$) is calculated according to Eq. 2.2 and depends only on the ratio of intra/extracellular concentrations of the free form of the acid.

$$\Delta G_{Tr,AH} = R \cdot T \cdot ln \frac{C_{AH,e}}{C_{AH,i}} \qquad \text{Eq. 2.2}$$

where $C_{AH,i}$ and $C_{AH,e}$ are the intra and extracellular (in the medium) concentrations respectively of the free form of the acid *AH*. Note that $C_{AH,e}$ depends on the net production rate of this product and on the reactor pH.

The energy as ATP required/obtained from transport processes is calculated by addition of the energy changed of all actively transported species (see Eq. 2.3) and it contributes to the flux *v8* from the bioreaction network (see Figure 2.1) i.e. the ATP hydrolysis for transport and maintenance purposes.

$$ATP_{Tr} = \sum_{j} \frac{\Delta G_{Tr,j}}{\Delta G_{ATP}} \cdot V_{Tr,j} \qquad \text{Eq. 2.3}$$

where $\Delta G_{Tr,j}$ is the Gibbs free energy change of the transport outwards of one mol of species *j*; $V_{Tr,j}$ is the total flux of acidic species transported and ΔG_{ATP} is the Gibbs free energy change of the ATP hydrolysis (i.e. flux v_8 from the bioreaction network).

The amount of acid needed to be actively transported is the sum of the amount produced plus the amount freely diffused inwards (see Figure 2.3). The free diffusion term of an acid into the cell (in $mol_{AH}/L \cdot h$) is calculated according to Eq. 2.4.

$$V_{bdiff} = Diff_{AH} \cdot (C_{AH,e} - C_{AH,i}) \cdot X \qquad \text{Eq. 2.4}$$

where $Diff_{AH}$ ($L/mol_X \cdot h$) is a diffusivity parameter for the acid through the membrane and X is the biomass concentration in the reactor (mol_X/L). Thus the total amount to be transported is $V_{Tr,i} = \tau_i + V_{bdiff}$ where τ_i is the net metabolic production of the species *i* (see Figure 2.1).

The sum of all the energy requirements for transport (ATP_{Tr}) is added to the maintenance term in ATP equivalents (ATP_m) and provides the total flux of ATP hydrolysis (Eq. 2.5). ATP_m is calculated from the parameter ΔG_m that is the amount of free energy consumed for maintenance per unit of biomass and time (Tijhuis *et al.*, 1993).

$$v_8 = ATP_{mTr} = ATP_m + ATP_{Tr} \qquad \text{Eq. 2.5}$$

The diffusion terms and the energy involved in transport are dependent on the concentration gradient and therefore the values of the internal concentrations of metabolites must be defined.

2.2.4. Intracellular metabolite concentrations

Intracellular metabolite concentrations are needed to calculate the energy requirements for transport (see Eq. 2.2 and Eq. 2.4). Some of the intracellular concentrations are given as parameters and/or directly determined by the environmental conditions (e.g. NADH/NAD by the hydrogen pressure as explained below). The estimation of intracellular concentrations is based on the assumption that microbial cells tend to operate in a steady state regime where a minimum energy leakage occurs. This is equivalent to assume a maximum energy recovery from transport processes or an optimal energetic performance (Beard *et al.*, 2002). The intracellular concentration values taken for the acid species are assumed to be the largest possible while still fulfilling physiological homeostasis, as well as mass transfer and thermodynamic limitations.

1. The intracellular metabolite concentrations are constrained by physiological maxima (values provided as parameters) to maintain cell homeostasis, $maxC_{i,A}$ values of the dissociated forms are used (Table 2.1).

2. The intracellular concentrations of acids cannot exceed a limit value that equals the acid free diffusion outwards to the net product formation flux (see Eq. 2.4). This would lead to energy consumption for active product uptake which is not realistic. This diffusional limited maximum concentration of intracellular product (undissociated form) is calculated according to Eq. 2.6.

$$C_{AH,i}^{DiffLim} = C_{AH,e} \cdot \frac{v_{AH}}{Diff_{AH} \cdot X} \qquad \text{Eq. 2.6}$$

The concentration of the dissociated form is given by the constant intracellular pH.

3. Finally intracellular concentrations of acid products must be low enough to enable thermodynamic feasibility of their bioreaction pathway (Eq. 2.1). A thermodynamic feasible maximum concentration can be calculated.

The smallest of the three limit intracellular concentration values fulfils the three conditions and is taken as the intracellular concentration of the undissociated form of the acid product.

2.2.5. Parameters and inputs of the model

The following parameters and inputs are required in the current version of the model, Table 2.1 shows the values adopted for the model parameters.

i) Constant *intracellular pH* value (pH_{int}) of 7 is assumed. A constant pH of the cytoplasm is generally considered necessary for cellular homeostasis, even though in the literature (Diez-Gonzalez and Russell, 1997) small variations in the intracellular pH have been reported.

ii) A *minimum Gibbs free energy change* (ΔG_{min}) is defined for all bioreactions. This assumption is based on the consideration that there should be a minimum driving force to enable a flux through the enzymatic network. This is a crucial parameter related for the hydrogen pressure effect on the product spectrum.

Table 2.1. Physiological parameter values used.

Parameter	Value	Units
pH_{int}	7.0	Ø
Ci_{Pyr}	7.5	mM
Ci_{AxP}	3.0	mM
Ci_{Pi}	10.0	mM
ATP/ADP	2.67	M_{ATP}/M_{ADP}
$maxCi_{Lac}$	10	mM
$maxCi_{Bu}$	10	mM
$maxCi_{Pro}$	10	mM
$maxCi_{Ac}$	10	mM
$Diff_{LacH}$	100	$1/(M_X \cdot h)$
$Diff_{BuH}$	85	$1/(M_X \cdot h)$
$Diff_{ProH}$	100	$1/(M_X \cdot h)$
$Diff_{AcH}$	123	$1/(M_X \cdot h)$
ΔG_{min}	-5	kJ/mol
ΔG_m	5.24	$kJ/mol_X \cdot h$

iii) The *intracellular pyruvate concentration* is predefined since it cannot be calculated because the irreversible glycolysis is not explicitly modelled. A value taken from literature reports (Yang *et al.*, 2001) is used. The role of pyruvate as central branch point makes its

concentrations effect important in terms of the hydrogen level at which thermodynamic limitations occur and therefore changes in the product spectrum. Its effect is strongly related to the value of ΔG_{min} used.

iv) Total *intracellular concentration of ATP+ADP* and their *concentrations ratio* (from Thauer *et al.*, 1977) as well as the *phosphate concentration* (P_i) (proposed by Schink, 1997) are given. These parameter values determine the Gibbs free energy change of the ATP hydrolysis inside the cell.

v) *Intracellular dissociated product concentrations* are restricted by *a physiological maximum*, probably set by the osmotic pressure that can be tolerated by the cellular membrane. Reasonable values in the order of magnitude of those reported in the literature (Diez-Gonzalez and Russell, 1997) were taken.

vi) The *diffusivities of the undissociated and active-transported acids* through the cellular membrane are predefined. This largely unknown parameter is roughly estimated from literature information from the relation between maximum rates and affinity constant values, assuming that kinetics are limited by diffusion at very low concentrations $Diff \approx q_S^{\max} / K_S$. The relation between the diffusivities of the three volatile fatty acids is taken from (Xiang and Anderson, 1998).

vii) Energy *dissipation for maintenance* purposes (ΔG_m) is estimated from a temperature dependent generalized correlation (Tijhuis *et al.*, 1993; Heijnen, 1999).

The *overall glucose uptake* rate eliminates one degree of freedom of the biochemical network. Some other operational inputs are provided as the dilution rate of the reactor and the substrate concentration in the feed stream.

2.2.6. Maximum growth criterion

After defining the model inputs and parameters the remaining system consists of a network with 4 degrees of freedom. The assumption that the mixed culture will establish product fluxes that provide the maximum growth rate (Hellingwerf *et al.*, 1982) leads to the possibility of defining an optimisation problem (see Eq. 2.7). By maximisation of the biomass production rate, the optimum vector of fluxes and fermentation products can be

obtained. The fluxes selected for maximisation of the optimisation criterion are *v2*, *v3*, *v4* and *v5* corresponding to the fluxes of butyrate, propionate, acetate and lactate production respectively. The flux *v9* of the anabolism leading to biomass growth can subsequently be calculated and maximized (see Figure 2.1).

$$\max v_9 = f(v_2, v_3, v_4, v_5) \qquad \text{Eq. 2.7}$$

2.2.7. Operational variables

The selection of environmental variables to be studied was based on their relevance, according to literature, for the product spectrum. The effect of the following process variables on the product formation is evaluated:

i) ***The hydrogen partial pressure (P_{H2})*** has an important effect on the thermodynamic feasibility of certain reactions and influences the product spectrum obtained (Mosey, 1983; Ruzicka, 1996). Hydrogen will affect the oxidation state of the electron carrier in the system (NADH). Thus high hydrogen partial pressures will thermodynamically limit oxidative pathways like acetate production. Supplying an MCF-system with variable amounts of inert gas, like molecular nitrogen, can be used to manipulate the hydrogen pressure in the system.

ii) ***The reactor pH*** has been identified as an important variable in glucose fermentations (Zoetemeyer *et al.*, 1982b). The pH will have a strong impact on transport energetics of undissociated organic acids that will dissipate the proton motive force and freely diffuse into the cell at low pH-values.

iii) ***Substrate concentration***. Elevated substrate concentrations will lead upon conversion to elevated product concentrations. At high product concentrations individual reactions will become less favourable and active transport more energetically expensive, suggesting that a wider product spectrum will be obtained.

2.3. Model implementation and computation

2.3.1. Optimisation of biomass growth

The model evaluation consists of calculating the feasible flux vector that provides the maximum biomass yield for the actual environmental conditions. The biomass growth is limited by energy availability as ATP.

The energy coupled transport processes included in the model led to a non-linear system unable to be solved by linear programming and therefore alternative optimisation algorithms are used. To find the global optimum for a maximum biomass yield, direct search optimisation methods were applied. A random search algorithm was initially used, looking for the widest search region (Rodríguez and Carrasco, 2002), followed by a Simplex method (Nelder and Mead, 1965), faster in final convergence.

The results obtained showed however that, even with these computationally demanding methods, local suboptima were often achieved. This is attributed to the fact that under certain environmental conditions the biomass yield is almost insensitive to relevant changes in the product spectrum. To overcome this drawback a partially heuristic optimisation method was designed for this particular problem, based on assuming equal energy yields obtained per pyruvate converted in the different metabolic pathways.

To obtain a proper initial estimate for the optimal flux vector and avoid local suboptima, for each environmental condition, the net energy as ATP obtained per mol of pyruvate vs. the percentage of catabolic pyruvate processed through each product pathway (using Eq. 2.2, Eq. 2.3 and Eq. 2.4) is calculated.

These energy yields at pH 6 and very low (non limiting) hydrogen pressure for each pathway are shown in Figure 2.4. This figure shows how the energy obtained from each acid product pathway starts at the minimum flux possible around the number of ATP obtained by substrate level phosphorylation (see Figure 2.1) and changes when the flux of product increases. This is due to the accumulation of product which leads to a pH dependent energy cost/production by active transport.

The quasi optimum will correspond to the highest energy level (dashed line in Figure 2.4) at which 100% of catabolic pyruvate is used. Note that a maximum of only 50% of the available pyruvate can be directed into the butyrate pathway because the other 50% must go through the acetate pathway since one acetate is required per butyrate produced (see Figure 2.1).

By using this method for choosing the quasi optimum initial vector of the optimisations, global optima are easily reached by the Simplex (Nelder and Mead, 1965) algorithm in a much more reliable and computational efficient way.

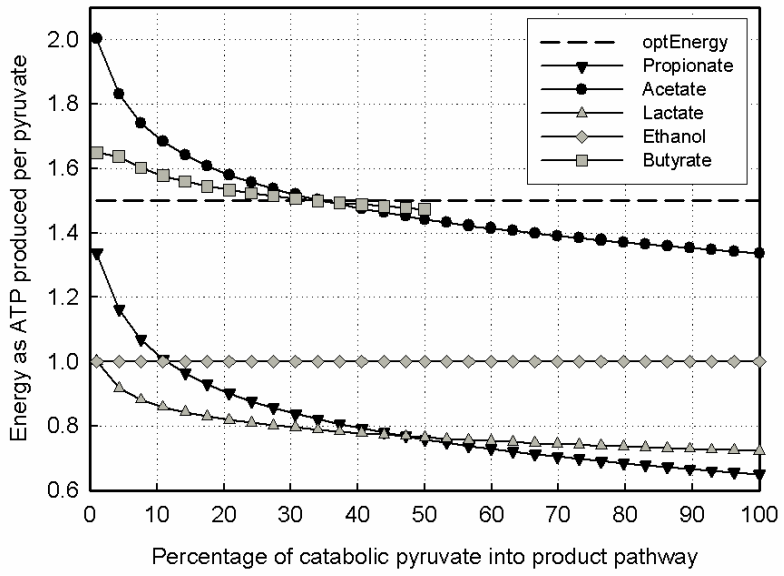

Figure 2.4. Energy as ATP obtained per mole of pyruvate vs. the percentage of catabolic pyruvate directed into the product pathway (at pH 6 and very low hydrogen pressure).

The reason for conducting a subsequent Simplex optimisation and not assuming the quasi optimum as global optimum is because there are interactions among the energy yield functions of the different products (Figure 2.4) that had to be neglected to enable their use to calculate that quasi optimum. Particularly there is an interaction between the thermodynamic limits of the butyrate and acetate pathways where acetate is substrate and product respectively.

2.3.2. Model evaluation procedure

Figure 2.5 shows a scheme of the model evaluation procedure. An estimated biomass yield is initially taken and together with the glucose uptake flux (provided as parameter) a pyruvate flux to catabolic products is defined.

For the given environmental conditions the energy yield functions as in Figure 2.4 are calculated and the energetic optimum flux vector is calculated as explained above. This quasi optimum vector is the initial point for the final optimisation of the biomass yield. This direct search final optimisation evaluates different vectors until the convergence criteria are fulfilled. The evaluation of the metabolic network for a given flux vector is detailed in Figure 2.5.

Once defined the degrees of freedom of the metabolic network, the net formation of all products (transport fluxes τ_j) and the extracellular concentrations of products can be calculated by application of the mass balance equation of the reactor. A chemostat reactor is used in this case (Eq. 2.8).

$$C_j = \frac{1}{D} \cdot \tau_j + C_{j,feed} \qquad \text{Eq. 2.8}$$

where D is the dilution rate (h^{-1}); and $C_{j,feed}$ is the concentration of the species *j* in the feeding stream (only glucose and ammonium are present in this case).

The extracellular concentrations of the undissociated forms of the transportable acids are calculated from the reactor pH and the dissociation constant of the acid. Intracellular concentrations, calculated from the model parameters as explained previously, together with the reactor concentrations, enable the calculation of the energy requirements for transport ($v8$) from Eq. 2.3 to Eq. 2.5.

The flux pattern is finally checked for thermodynamic feasibility of all the bioreactions and returned to the optimisation algorithm.

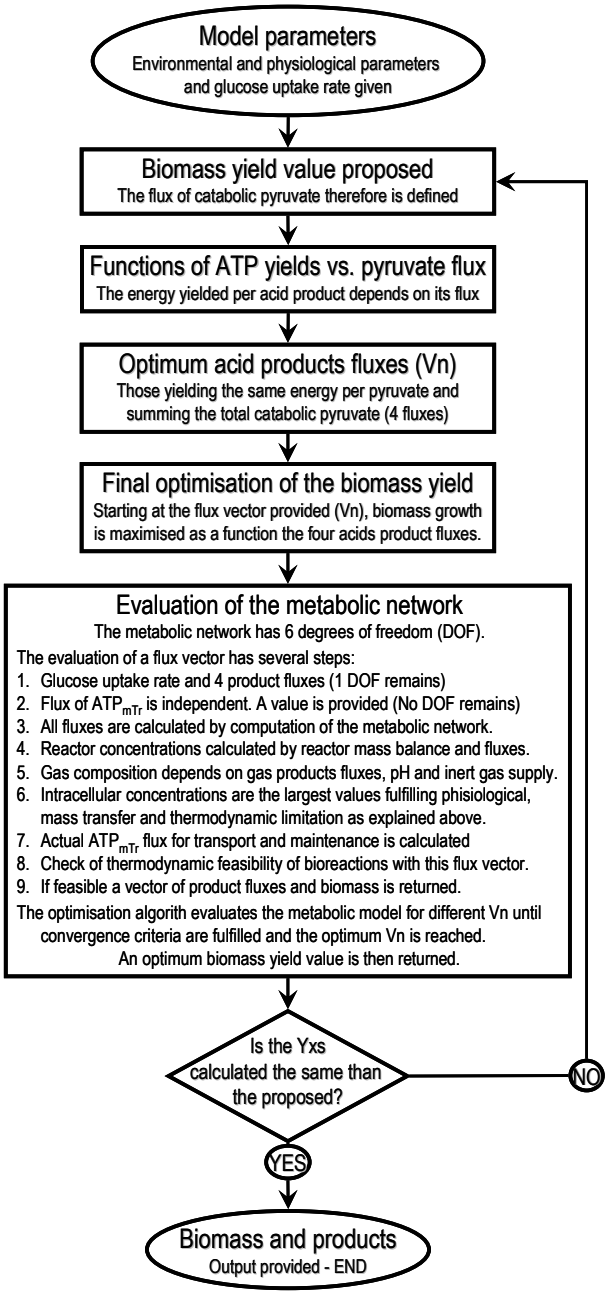

Figure 2.5. Scheme of the model evaluation procedure.

2.4. Model results

The effects of the operational variables considered are studied relative to a reference operation defined in Table 2.2. The effects of the pH, P_{H2} and substrate concentration are studied separately by individually changing these parameters. Each condition simulated results from a global optimisation of the biomass production for the given operational conditions.

Table 2.2. Reference operational conditions.

Condition	Value	Units
Dilution rate (D)	0.125	h^{-1}
Reactor pH (pH_{ext})	6.25	Ø
Inert gas flow rate (Q_{IG})	80.0	$L/mol_X \cdot h$
Substrate concentration ($CGlu_{inf}$)	0.056	mol_{Glu}/L
Ammoniun in the influent ($CNH4_{inf}$)	0.056	mol_{NH4}/L
Temperature (T)	30.0	ºC
Total pressure (P_T)	1.00	atm
Biomass in the reactor (X)	0.045	mol_X/L

2.4.1. Effect of the hydrogen partial pressure

The hydrogen partial pressure has been identified as an important variable for determination of the product spectrum from glucose fermentation (Ruzicka, 1996; McCarty and Mosey, 1991; Inanc *et al.*, 1999). The hydrogen levels affect the NADH/NAD ratio and therefore the thermodynamic feasibility range of certain pathways.

The effect of the hydrogen pressure (P_{H2}) is considered by assuming the reaction $NADH + H^+ \rightarrow NAD^+ + H_{2(g)}$ very close to equilibrium with a ΔG value given by the parameter ΔG_{min} (see Table 2.1). Herewith the ratio $NADH/NAD^+$ becomes a function of the hydrogen partial pressure and the intracellular pH (assumed constant at pH 7) (see Eq. 2.9). The ratio $NADH/NAD^+$ affects all the redox reactions in the model since the *NAD* form is the electron carrier considered. High hydrogen partial pressures lead to a very high $NADH/NAD^+$ ratio making the oxidative processes (e.g. acetate formation) thermodynamically less favourable and, under certain conditions, not feasible (Mosey, 1983).

$$\frac{NADH}{NAD^{+}} = P_{H2} \cdot 10^{pH_{\text{int}}} \cdot \exp\left(\frac{\Delta G_{v7}^{0} - \Delta G_{\min}}{R \cdot T}\right) \qquad \text{Eq. 2.9}$$

The hydrogen partial pressure in the reactor can be manipulated by inert gas supply. This causes a dilution effect on the hydrogen partial pressure whose value depends not only on the hydrogen but also on the carbon dioxide production rate and the reactor pH that affects the gas/liquid phase distribution of each mol of CO_2 produced.

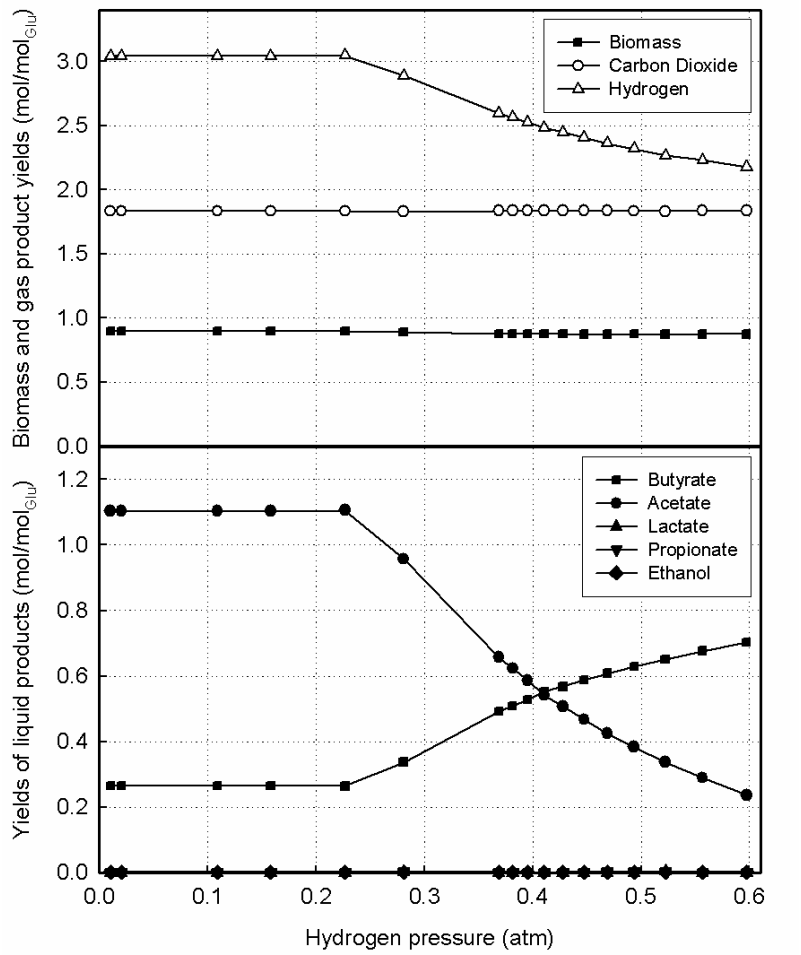

Figure 2.6a. Model prediction of the effect of the hydrogen partial pressure on the product spectrum at the operational conditions from Table 2.2.

Figure 2.6a presents the yield of products versus the hydrogen partial pressure at the reference operational conditions from Table 2.2. The energy as ATP obtained by each product is shown in Figure 2.6b. At low hydrogen pressure acetate is the main product. An increase in the hydrogen pressure (induced by less inert gas supply) causes a thermodynamic limitation in the acetate production reaction, forcing a decrease in its intracellular concentration and making its transport outwards more energetically expensive. This causes a shift to production of butyrate since hydrogen and acetate are incorporated per butyrate produced. The hydrogen production therefore decreases since formation of the more reduced butyrate yields no net hydrogen (see Figure 2.1).

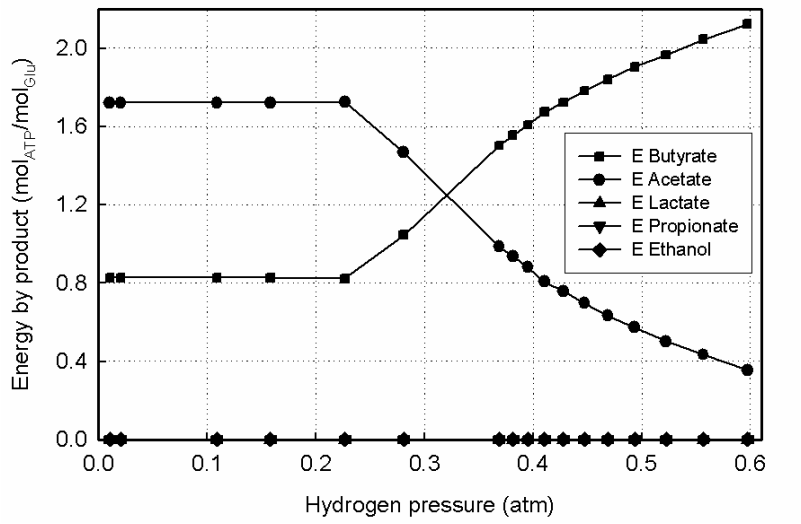

Figure 2.6b. Model prediction of the effect of the hydrogen partial pressure on the energy contribution by product at the operational conditions from Table 2.2.

The minimum Gibbs energy change (ΔG_{min}) was found to be a sensitive parameter defining the point at which the increase of hydrogen pressure reaches a thermodynamic limitation and causes a shift to products other than acetate. This makes the parameter (ΔG_{min}) suitable for identification.

2.4.2. Effect of the reactor pH

Figure 2.7a presents the product spectrum predicted by the model at different reactor pH-values for the conditions shown in Table 2.2. The main trends observed are related to the energetics of the transport processes and the free diffusion of the undissociated acids.

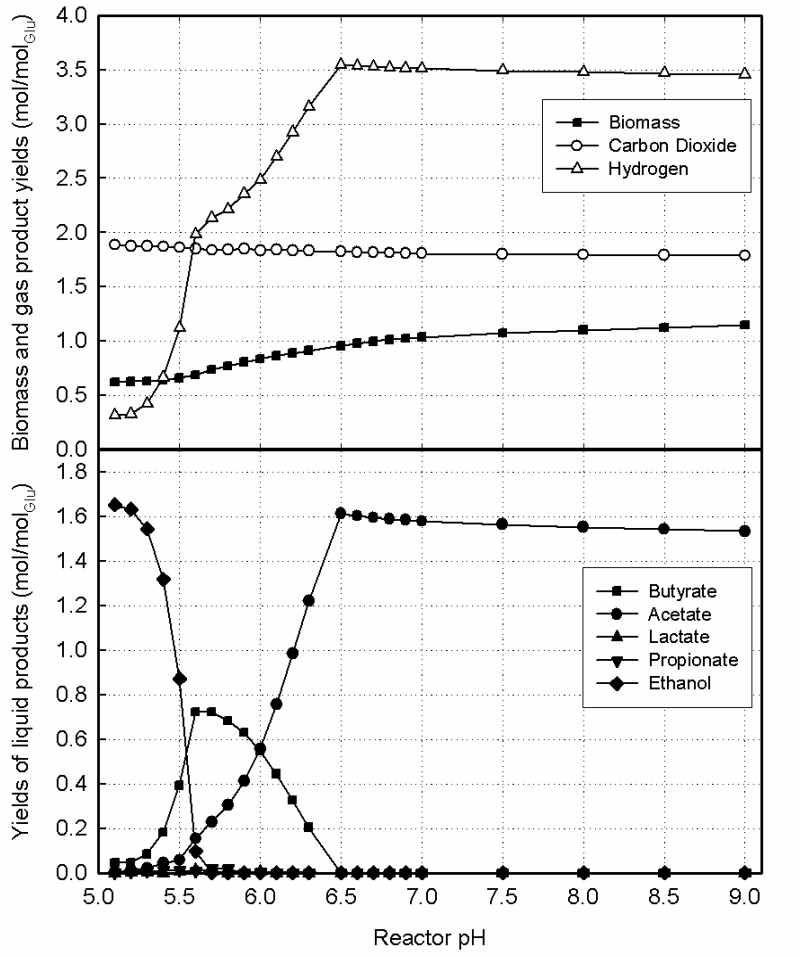

Figure 2.7a. Model prediction of the effect of the reactor pH on the product spectrum at the operational conditions from Table 2.2.

At high pH values acetate is predicted as the main product since it yields one extra ATP per pyruvate. The acetate production decreases at lower pH values since the concentration of the undissociated form of the acid increases, resulting in more energy requirements for outwards transport of acetic acid. Butyrate replaces acetate as main product at decreasing pH-values since the production of one butyrate incorporates one acetate (see Figure 2.1) and consequently less acid molecules need to be transported per glucose converted.

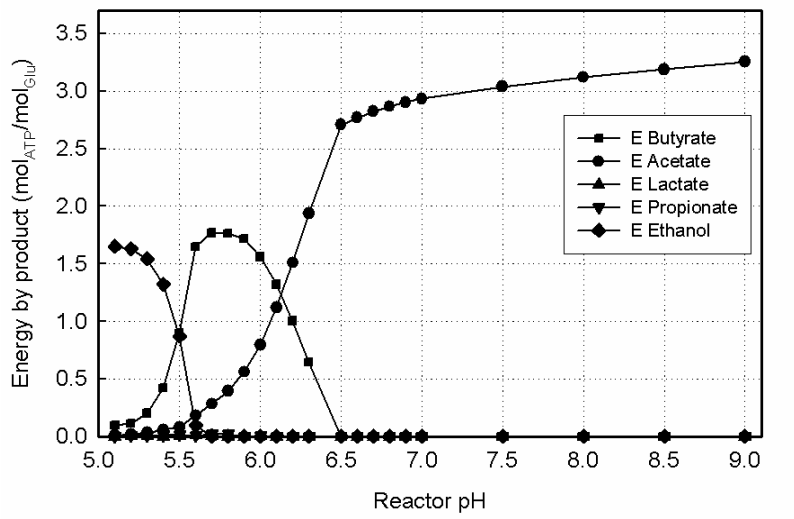

Figure 2.7b. Model prediction of the effect of the reactor pH on the energy contribution by product at the operational conditions from Table 2.2.

At lower pH values (< 5.6) the butyrate decreases as well, and ethanol becomes the dominant product. At these low pH values any acid transport outwards becomes energetically very expensive. The current model did not predict any propionate or lactate production, this is due to a too simple implementation of metabolic constraints in this initial model. Incorporation of CoA pathways and maybe other electron carriers will enable prediction of these products. An increasing biomass yield is predicted at higher pH values since extra energy is obtained from active transport of acids. Thermodynamic limitations are never reached by the biochemical pathways under these conditions, suggesting that product formation is primarily dependent on the membrane related processes.

Simulations were also conducted at different values of the diffusivity of the active transported acids through the cell membrane. These parameters are sensitive to the pH value at which the shift in the product formation occurs and appear therefore suitable for identification.

2.4.3. Effect of the substrate concentration

Figure 2.8a shows the effect of the feed substrate concentration on the product spectrum.

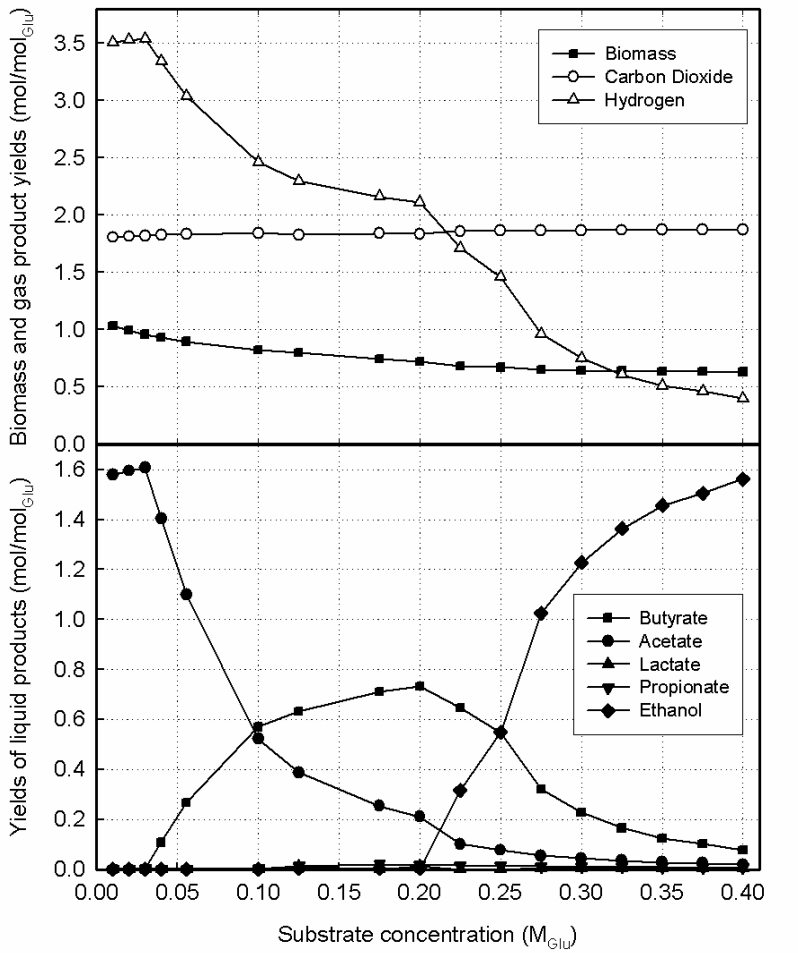

Figure 2.8a. Model prediction of the effect of the substrate concentration on the product spectrum at the operational conditions from Table 2.2.

The energy as ATP obtained by each product as a function of the substrate concentration is shown in Figure 2.8b.

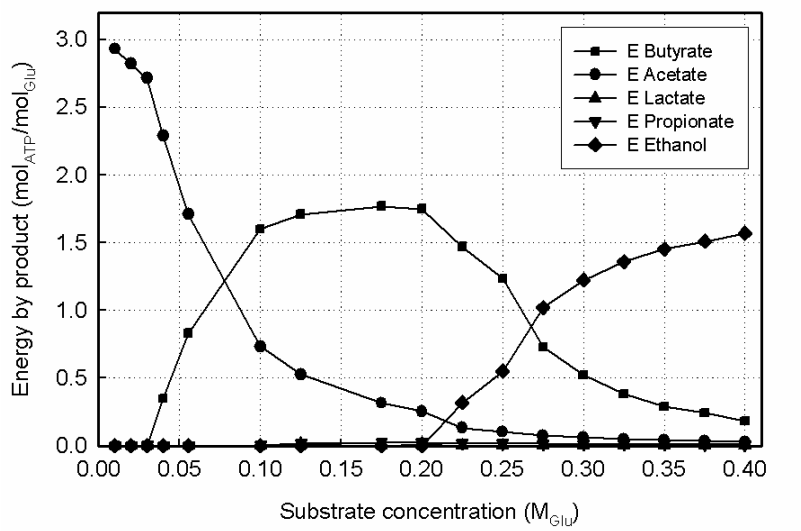

Figure 2.8b. Model prediction of the effect of the substrate concentration on the energy contribution by product at the operational conditions from Table 2.2.

In order to isolate as possible the effect of substrate concentration, the hydrogen pressure in this simulation is maintained at low levels (below 0.1 atm) by increasing the gas supply proportionally with the substrate concentration. This granted that the thermodynamic limit of any bioreaction is never reached due to high hydrogen pressures.

Higher substrate concentrations imply higher product concentrations in the chemostat reactor. The effect of the higher product concentrations in the reactor is therefore reflected as a higher energy requirement for transport of these products against a steep concentration gradient. The effect of increasing the substrate concentration showed a profile analogue to the pH-profile, shifting from acetate first to butyrate and then to ethanol as main products.

2.4.4. Combined effect of pH and hydrogen pressure

Figures 2.9a-b-c present some products and biomass yields as function of the hydrogen pressure and reactor pH. The results presented in Figure 2.9 have been slightly different computed by manually fixing the biogas composition to cover a wider range of hydrogen pressure and reactor pH values. The surfaces give an idea of the combined effect of both operational variables.

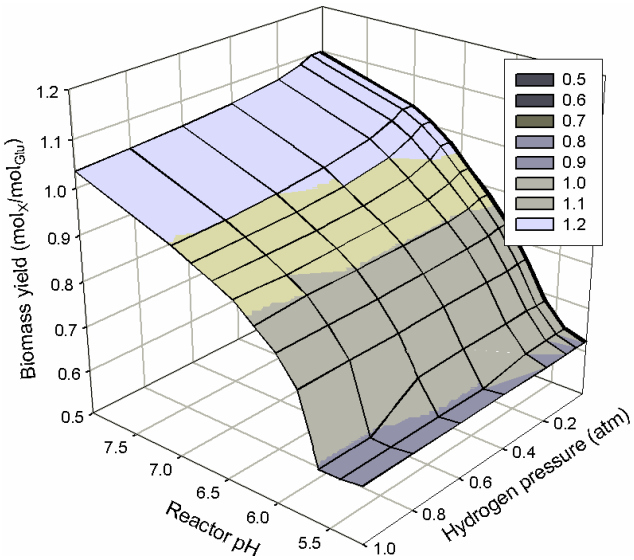

Figure 2.9a. Biomass yield vs. the reactor pH and hydrogen pressure. Data have been computed at a fixed P_{CO2} of 0.5 atm changing P_{H2} manually.

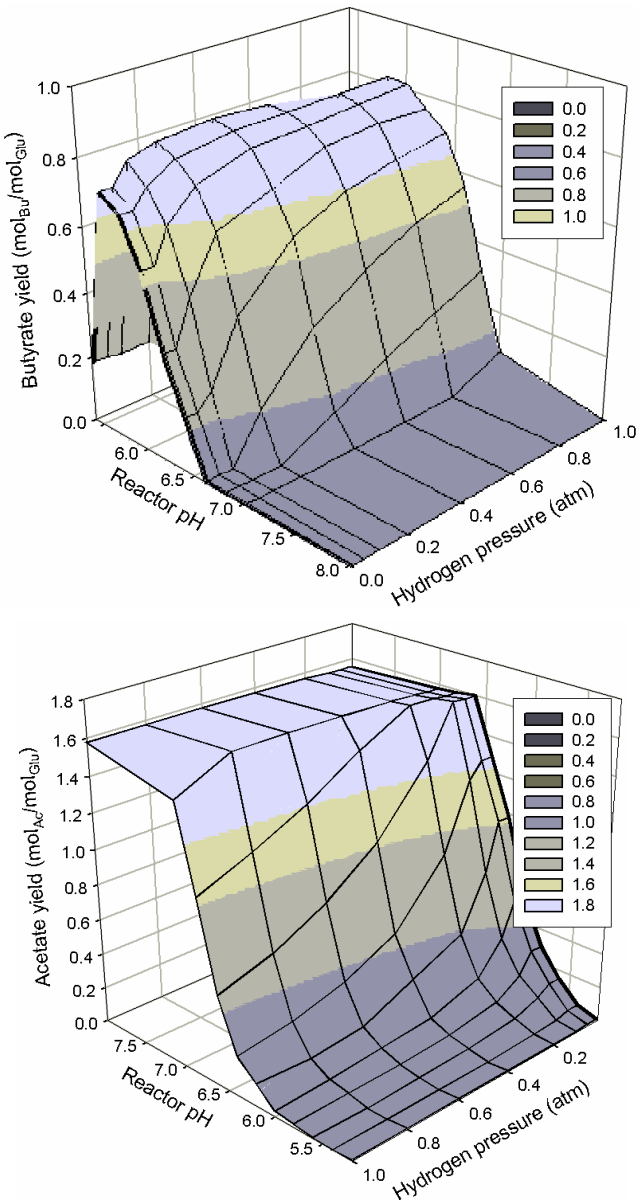

Figure 2.9b. Butyrate and acetate catabolic yields vs. the reactor pH and hydrogen pressure. Data have been computed at a fixed P_{CO2} of 0.5 atm changing P_{H2} manually. (Note the different axis layout).

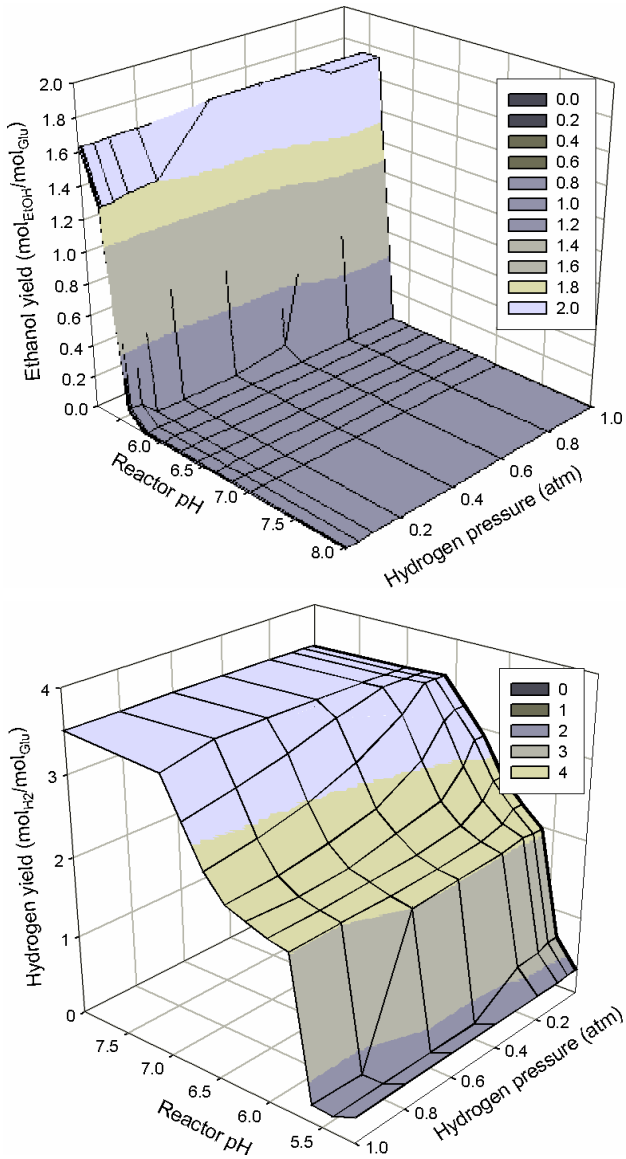

Figure 2.9c. Ethanol and hydrogen catabolic yields vs. the reactor pH and hydrogen pressure. Data have been computed at a fixed P_{CO2} of 0.5 atm changing P_{H2} manually. (Note the different axis layout).

2.5. Discussion

2.5.1. Model assumptions

The model developed is based on a number of assumptions that need to be justified. The model purpose of predicting the dependence of the fermentation product spectrum as a function of the operational conditions from a bioenergetic perspective must be considered for justification of the simplifications undertaken.

i) The fundamental hypothesis that a microbiological system is evolutionary optimized for maximal biomass production is frequently used in metabolic engineering for solving underdefined systems (van Gulik and Heijnen, 1995; Westerhoff, 1982).

ii) The minimal thermodynamic driving force assumption is defendable when dealing with anaerobic systems (Schink, 1997). This allows also the calculation of some internal concentrations of metabolites by assuming that a minimum energy loss occurs (Beard *et al.*, 2002; Kleerebezem and Stams, 2000).

iii) The neglection of microbial diversity by assuming a virtual microorganism able to carry out the most common fermentative conversions is acceptable in steady state conditions. An analogy between microbial and enzymatic conversions is established and enables the use of techniques developed in the metabolic modelling field.

iv) The model is applied to a homogenous reaction mixture where all bacterial cells are in suspension, it can be extended to a biofilm or flocculated system by implementing the model in a reaction-diffusion equation system.

v) Some minor products are not considered either for the sake of simplicity of the model or because they are produced in small amounts in most fermentations (e.g. propanol, acetone, butanol, etc) (Batstone *et al.*, 2002; Thiele and Zeikus, 1988).

vi) Some processes as the gas-liquid transfer of hydrogen or its formation from electron carriers are assumed to be in equilibrium. Deviations from equilibrium should affect the threshold values where the product shifts occur but not the qualitative behaviour.

vii) The same minimum Gibbs energy change value (ΔG_{min}) is assumed for all the reactions. Further development of the model could take different values for this parameter depending on the number of steps in each lumped catabolic reaction and/or the number of electrons transferred in each step.

viii) The full energy recovery assumed should not affect significantly the prediction of product formation (that is the model objective) but mainly the biomass yield predicted. This is justified for the sake of maintaining a reduced number of parameters in the model.

ix) The model assumes steady state at all time scales, for future application and integration with dynamic models, time scale related parameters need to be defined.

2.5.2. Preliminary results obtained

Experimental results from several MCF studies have been reported in the literature based on glucose (Zoetemeyer *et al.*, 1982b; Horiuchi *et al.*, 2002; Fang and Liu, 2002) and lactose (Fang and Yu, 2001; Yu *et al.*, 2004) fermentation. Experiments have been carried out under different conditions of pH and hydrogen pressure, but almost no uniform and reproducible product spectra as a function of the operational procedures could be identified. There is a major lack of experimental data in the literature obtained from steady state chemostat reactors, under very well controlled conditions, required for calibration and validation of this type of models. Still, the main experimental trend observed for acetate and butyrate, depending on the hydrogen pressure and the reactor pH, is qualitatively described by the model presented here. A shift from acetate to butyrate as major product when either pH decreases and/or hydrogen pressure increases is predicted.

No lactate or propionate were predicted by the current model version despite they appear experimentally. Future work is aimed at incorporating more biochemical information and enable prediction of these products.

2.5.3. Model identifiability

The value given to minimum Gibbs energy change required by the bioreactions (ΔG_{min}) has a major impact on the effect of the hydrogen on the product spectrum. The values of the diffusivities of the undissociated forms of the acids through the cell membrane have on the other hand a major impact on the effect of the reactor pH and substrate concentration on the product spectrum. This makes these two parameters adequate for independent identification. The importance of other parameters in the products prediction is rather smaller compared to these two. These facts led to a very simple model in terms of the reduced number of parameters to be identified, as a consequence of having defined the model mainly based on the application of fundamental laws, as mass and energy balances, together with the optimisation criterion proposed.

2.5.4. Modelling approaches

Existing mixed culture fermentation models with fixed stoichiometry require many parameters to describe the stoichiometry for all product formation pathways and biomass in addition to inhibition parameters for each conversion process. Nevertheless, these models are not able to reproduce some changes in the stoichiometry observed experimentally (Kleerebezem and van Loosdrecht, 2004). Moreover some of these models treat generally thermodynamic limitations as kinetic inhibitions. This may be a consequence of extrapolation to anaerobic systems of certain modelling principles as a legacy from modelling of aerobic systems that run under very different energetic conditions far from thermodynamic equilibrium. The other extreme, pure black box thermodynamic models, with only input and output fluxes defined using a global optimisation of Gibbs energy production, present also important disadvantages. The large number of degrees of freedom complicates the optimisation while the absence of biochemical information permits inexistent pathways or others known to have a different stoichiometry.

The modelling methodology presented here combines a quasi pure thermodynamic approach with a minimum biochemical information, resulting in a model with less degrees of freedom and a minimum number of relevant parameters. The model illustrates a methodology to estimate the stoichiometry of different products from glucose in a mixed culture. It could be integrated in classic dynamic models by changing the stoichiometry parameters for different conditions and including time-scale-related parameters.

Future work is aimed at structural validation of the model and incorporation of more biochemical information to intend to define the system in more detail to be able to predict a wider spectrum of products. Incorporation of CoA metabolites or the role of electron carriers other than NADH forms could be considered as well.

Experimental data from well controlled and reproducible experiments, in chemostat reactors in steady state under different operational conditions, is required to validate the model approach presented in this work and to define its range of applicability.

The use of mechanistic models should lead to better understanding of MCF processes and enable the design of much better control strategies. Improved performance of MCF will furthermore encourage the industrial fermentation of waste streams to produce valuable products as PHAs, solvents or hydrogen and extend the application of anaerobic digestion.

2.6. Conclusions

A modelling approach for mixed culture fermentations based on optimum energy exploitation in a metabolic network is proposed. A model, developed for prediction of the product formation under different conditions of pH, hydrogen partial pressure and substrate concentration, is presented to illustrate the modelling methodology proposed. The model predicts acetate as major product at low hydrogen pressure and neutral pH, while at lower pH and/or higher hydrogen pressure, butyrate becomes the major product. At lower pH values (<5.5) ethanol is predicted as the main fermentation product. Neither lactate nor propionate appear in any case at the current stage of the model.

All the information available from fundamental laws of mass and energy conservation is used leading to a model with a reduced number of interpretable and identifiable parameters that presented relevant sensitivity on the model output. The minimum Gibbs energy change required by the bioreactions (ΔG_{min}) is the most sensitive parameter to predict the effects of the hydrogen pressure on the product spectrum and appears suitable for identification. The same applies for the diffusivities of the active transported acids ($Diff_{AH}$) that are the most sensitive parameters to predict the effects of the reactor pH and substrate concentration on the product spectrum and appear therefore also suitable for identification as well.

2.7. Nomenclature

pH_{int}	Intracellular pH
$C_{i,Pyr}$	Intracellular concentration of pyruvate (mM)
$C_{i,AxP}$	Intracellular concentration of ATP plus ADP (mM)
$C_{i,Pi}$	Intracellular concentration of phosphate (mM)
ATP/ADP	Intracellular concentration ratio ATP/ADP (M_{ATP}/M_{ADP})
$maxC_{i,Lac}$	Physiological maximum intracellular conc. of dissociated lactate (mM)
$maxC_{i,Bu}$	Physiological maximum intracellular conc. of dissociated butyrate (mM)
$maxC_{i,Pro}$	Physiological maximum intracellular conc. of dissociated propionate (mM)
$maxC_{i,Ac}$	Physiological maximum intracellular conc. of dissociated acetate (mM)
$Diff_{LacH}$	Diffusivity of lactic acid through the cell membrane ($1/M_X \cdot h$)
$Diff_{BuH}$	Diffusivity of butyric acid through the cell membrane ($1/M_X \cdot h$)
$Diff_{ProH}$	Diffusivity of propionic acid through the cell membrane ($1/M_X \cdot h$)
$Diff_{AcH}$	Diffusivity of acetic acid through the cell membrane ($1/M_X \cdot h$)
ΔG_{min}	Minimum Gibbs energy change required by a bioreaction (kJ/mol)
ΔG_m	Gibbs energy consumed for maintenance purposes ($kJ/mol_X \cdot h$)
ATP_m	ATP consumed for maintenance purposes ($mol_{ATP}/mol_X \cdot h$)
ATP_{mTr}	ATP consumed for both maintenance and active transport ($mol_{ATP}/mol_X \cdot h$)
v_{AH}	Net production of the acid product AH ($mol_{AH}/L \cdot h$)

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Chapter 3

PROSPECTIVE OF MIXED CULTURE FERMENTATION MODELLING

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PROSPECTIVE OF MIXED CULTURE FERMENTATION MODELLING

Contents

Abstract

Some of the key issues of MCF modelling recently identified are discussed in this chapter. Based on the MCF model described in the previous chapter, its objectives and limitations will be identified. Possible improvements of the model are explored to assess these limitations including the validity of the assumptions made. Incorporation of kinetic processes, extension of the metabolic model with other electron carriers and other species are explored and preliminary results and discussion presented.

3.0. Summary

In this chapter an overview of the current situation and prospective of mixed culture fermentation modelling is provided. The objectives of MCF modelling identified focus on the prediction of the product formation in steady and dynamic state and on the mechanistic explanation of the phenomena associated with product formation.

In order to propose methods for model improvement, the major limitations of the existing MCF model are explained. The validity of assumptions like the global optimization criterion, based on the optimised bioenergetic performance, or the simplification of the mixed culture into a lumped metabolic network, are points of discussion. The assumption of optimum performance in steady state and the computational difficulties are also identified. The observation that the existing model is unable to predict formation of some products, observed in practice in certain conditions, is addressed. The omission of some species and processes of relevance is also identified.

An extensive metabolic network is presented to serve as framework for model upgrades including detailed pathways from pyruvate, as branch intermediate, to the final fermentation products to be excreted.

Different methods for assessment of the model limitations identified are explored at different levels of detail. In particular the metabolism and physiology associated issues are investigated and discussed in more detail. Special attention is given to the potential role of formic acid as regulation species and to the possible kinetic limitations induced by very low intermediate concentrations within reaction pathways due to limitations in the cofactor concentrations.

Based on the experience and the results from the exploration of alternatives, a route plan for improvement of MCF models is proposed by balancing difficulty of implementation, expected relevance for the model results and level of uncertainty.

3.1. Introduction

Mixed culture fermentation processes aim for conversion of low cost feedstocks (e.g. from agroindustry) into valuable products, including building-blocks for the chemical industry and energy streams such as hydrogen and ethanol.

The main technological challenge in mixed culture fermentation is to control the product spectrum formed. Fermentations tend to produce mixtures of products, which have similar properties and are therefore difficult to separate. Currently, this is mainly overcome by the use of pure or purified substrates and sterile conditions enabling the cultivation of homofermentative strains. Major limitation of the use of pure cultures is the increase of the fermentation costs. Mixed culture fermentation processes have the possibility of reducing the operational costs (and hence, capital costs) significantly since sterile conditions are not required and operation is generally more straightforward. In particular, viability of low value, high volume products such as ethanol, organic acids and hydrogen is very sensitive to processing cost and mixed culture fermentation (MCF) is an economically attractive process, provided that a high product yield can be established.

Since the product spectrum depends strongly on the cultivation conditions in MCF processes (Horiuchi *et al.*, 2002; Zoetemeyer *et al.*, 1982), it is possible to direct MCF to produce specific products by manipulation of these environmental conditions. For example, production of hydrogen is energetically more favourable if the hydrogen partial pressure is low, while production of ethanol is very sensitive to the pH value. This is the principle used in attempts to optimise microbial production of hydrogen, which is only economically viable as a MCF process (Benemann, 1996). However, despite the detailed understanding of the individual processes in pure cultures, as well as the good qualitative understanding of regulation processes, there is no generally accepted quantitative model for fermentation regulation from carbohydrates in mixed cultures. There are several reasons for this: (i) previously proposed models are fundamentally limited in a number of ways with incorrect or empirical basis and limited or impossible pathways, (ii) the models do not predict all (or even most) common scenarios and are simply bimodal and (iii) experimental data is limited in terms of design and outputs. Lack of a widely accepted fermentation model is of critical importance given the intense research and activity in commodity bioproducts and bioenergy.

In Chapter 2 of this thesis a "next generation" MCF model has been presented (Rodríguez *et al.*, 2006). The model applies fundamental laws of thermodynamics to evaluate favourability of individual pathways (including cell yield), for the current environmental conditions. The approach is supported by the fundamental hypothesis that natural selection, as imposed by evolution, selects the microbial populations that maximise their growth efficiency under the given environmental conditions.

This chapter deals with the investigation of alternatives to improve MCF models. Some of these alternatives have been explored and discussed in more detail.

3.2. Objectives of MCF modelling

The motivation of modelling mixed culture fermentations comes from the wish to control the process by understanding the metabolic regulation of the microbial system. MCF modelling aims at controlling the product formation by describing the product regulation mechanisms as a function of the operational conditions. The main possible objectives of MCF modelling would be:

1. Prediction of the product formation in steady state as a function of controllable operational variables like pH, partial pressure of gas products, substrate concentrations.

2. Prediction of transient products formation in dynamic situations as a function of the environmental conditions.

3. Mechanistic explanation of the phenomena involved in MCF and identification of the key regulation mechanisms of product formation and their limiting steps.

These three objectives have immediate process engineering implications. An MCF model for steady state would predict the formation of steady state products and assist the design of a continuous MCF process. However, the experimental information available up to date indicates that a true steady state in the culture performance could be difficult to obtain.

The second modelling objective addresses the formation of transient products occurring in dynamic situations. Some products are produced only under stress or perturbed situations and they can also be of interest. A reliable model identifying the mechanistics of these transitions would provide invaluable information to design dynamic operation strategies with the variables targeting these products.

The mechanistic explanation of MCF and identification of the controlling phenomena would provide new insights on these microbial systems and may help to elucidate which processes are controlled by thermodynamics, kinetics, ecological issues, etc. This provides an immediate engineering tool for direct assessment of the limiting steps of the desired conversions or for repressing the undesired ones.

3.3. Limitations of the existing MCF model

The metabolic network approach for MCF modelling presented in Chapter 2 of this thesis has a high potential for achieving the three modelling objectives mentioned above. This first MCF model was developed focusing on the first objective of a steady state prediction of products (Rodríguez *et al.*, 2006). The model predicts the product spectrum as a function of the pH, hydrogen partial pressure and substrate concentration in steady state. However, at the current stage the model needs experimental validation. Furthermore, preliminary experimental results (Temudo, 2005) have demonstrated that the model does not adequately predict product formation in all conditions. Experiments demonstrated stable ethanol production at high pH values whereas the model predicts acetate production.

Basic hypotheses of the model are that the mixed culture will behave optimally from an energetic efficiency point of view. It provides a product spectrum as a function of operational conditions by just maximising the biomass growth limited by energy availability. These assumptions provide a mechanistic interpretation to the model results. The existing model is a largely conceptual version and should be regarded as a first effort to describe the MCF process.

This biomass growth optimization is based on a very limited number of optimization criteria and consequently presents many limitations. These limitations are due to biochemical and physiological uncertainties and simplifications:

1. The hypothesis of optimal energetic performance is a defendable starting point, but it is likely that product formation is governed also by other factors, besides the optimal thermodynamic efficiency. This is even more likely considering that the differences in energetic yield as a function of the products formed is limited, since a dominant amount of ATP is always formed by the common glycolysis reaction. The only thermodynamic and not kinetic limitations assumed may very well be incomplete as well.

2. The chemical conversion reactions in the mixed culture are approximated by a metabolic network of a virtual microorganism and therefore assumed to behave as a single microbial species. This can be acceptable when the energetic efficiency hypothesis is true and when the bioconversions proceed close to thermodynamic equilibrium. If this is case the boundaries provided by thermodynamics largely define the energetics of the system and the product spectrum. It can happen however that the competence between different organisms depends on other issues than the product formed, like the affinity for substrates, and therefore the product spectrum would be the result of different catabolic capacities.

3. A true steady state performance is assumed by the model, but it is not clear for these systems that they are always operated at the energetically most efficient possible stable steady state. Experimentally the operation could be within the basin of attraction of a suboptimum steady state and not within the optimum predicted by the model. Thus other suboptimal product spectrum could be experimentally observed. The mechanisms of the transitions from a steady state with a product spectrum to another are unknown if exist.

4. Steady state fermentation systems are not frequently found in nature and this could mean that not enough evolutionary optimisation of the microbial species under steady state occurred yet. This possibility is another point to consider and therefore more detailed investigation of the transient regulation mechanisms becomes necessary.

5. The model predicts the product spectrum through an optimisation of the biomass yield, but this optimisation is currently very computing demanding and provides not always the global optimum sought.

6. The current model cannot predict some products experimentally observed or predict others under environmental conditions very different from reality.

7. The role of certain species seems to be underestimated and they should be considered in the model. Other processes and also possible kinetic limitations are not present in the current model and they could be responsible for the unpredicted formation of certain products observed experimentally.

In this chapter key issues are identified to asses the model limitations and diverse alternatives for future work are explored and discussed.

3.4. Extensive metabolic network of MCF

During the development of the existing MCF model the most common pathways for fermentation of carbohydrates were inventoried. The metabolic network used in the MCF model described in Chapter 2 is an additional simplification of this inventory that lumps serial reactions into one single step reactions. A more comprehensive metabolic network including the most frequent fermentation pathways is presented in Figure 3.1. The inventory of reactions was derived from several literature sources (Bückel, 1999; Madigan *et al.*, 2003; Kanehisa *et al.*, 2006; Kleerebezem and Stams, 2000; Moat *et al.*, 2002).

This metabolic network is used as reference for expanding the model since it includes detailed description of the fermentation pathways including intermediate metabolites. The role of electron carriers as FAD, Fd or NADP will be addressed later, here only NAD is considered for simplicity. While the glycolysis remains lumped, the pathways from pyruvate to the final products are detailed with all the intermediates involved. Extracellular species appear within a circle and intracellular within a square. The transport processes involving energy exchanges are not presented in the figure. The most important and well known pathways leading to each product are used but for some products different parallel pathways are possible, as is the case for propionate formation.

An important route for pyruvate conversion is an activation and decarboxylation producing formic acid and acetyl-CoA. The acetyl-CoA is a high energy central metabolite for the formation of acetate, butyrate and ethanol in bacteria. Deactivation of acetyl-CoA through acetyl-P yields 1 ATP by substrate level phosphorylation (SLP) and produces acetate as final product, with the highest yield of 1 ATP per pyruvate via SLP. When 2 acetyl-CoA combine into acetoacetyl-CoA and subsequently reduced to butyrate that is obtained by deactivation of butiryl-CoA yielding 1 ATP by SLP. This pathway has a high energy jump reduction step from crotonyl-CoA to butiryl-CoA (Kleerebezem and Stams, 2000), whose energy can potentially be harvested by proton translocation and subsequent production of extra ATP. Another major product that can be obtained through deactivation and further reduction of acetyl-CoA is ethanol. In bacteria the formation of ethanol occurs through the acetyl-CoA pathway but yeast can produce ethanol by direct decarboxylation of pyruvate as well.

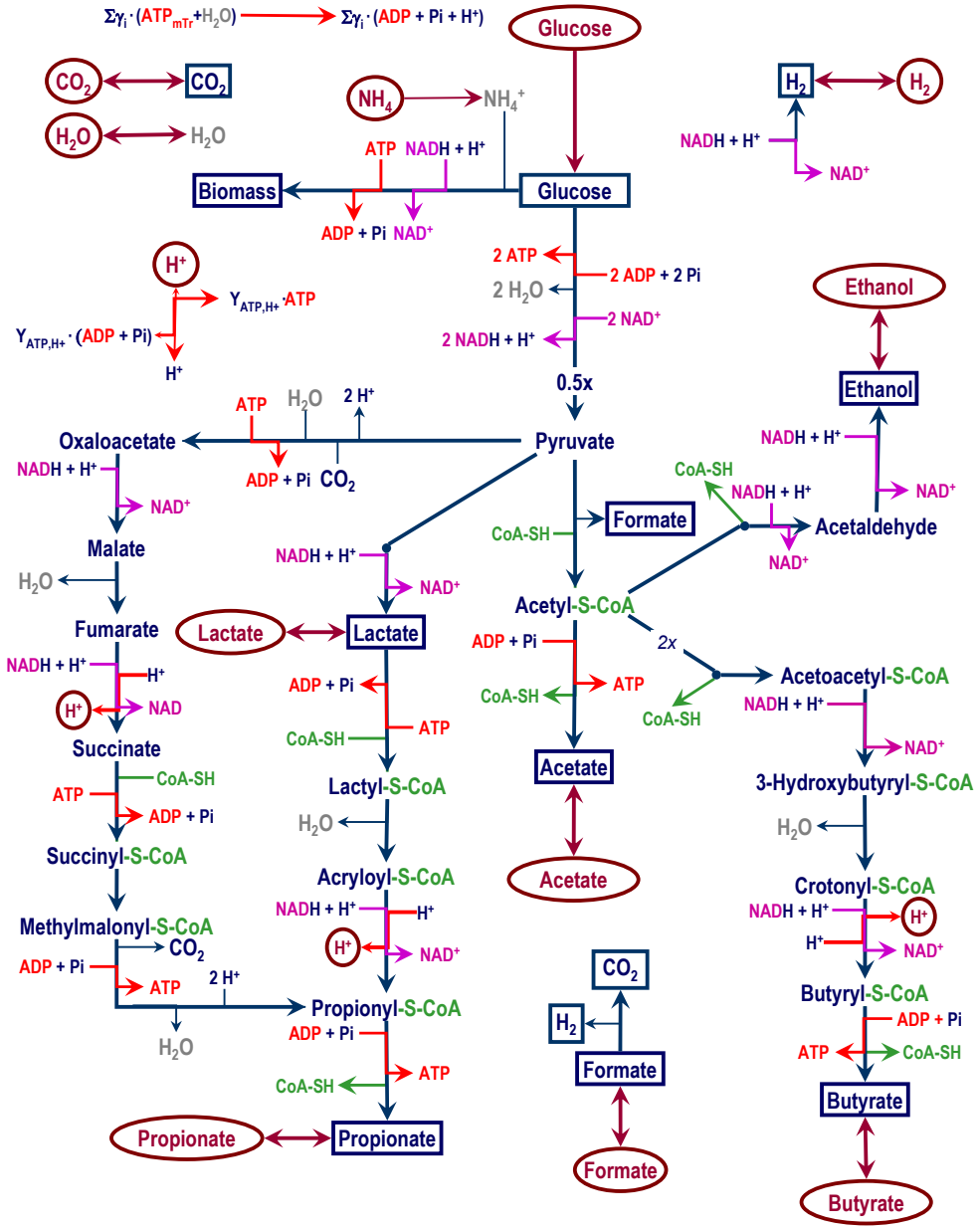

Figure 3.1. Metabolic network with the most common pathways for glucose fermentation. (NAD is assumed as the sole electron carrier with no FAD, Fd or NADP considered)

If pyruvate is not activated to a CoA-metabolite because not enough free CoA-SH is available in the cell or any other regulatory causes, it can the reduced to lactate as final product or proceed through some of the reactions of the TCA cycle reversed like the formation of oxalacetate and reduction to succinate. Succinate and lactate can be end-products but activation by CoA can lead to the production of propionate as well. Production of propionate is neutral in terms of ATP generation by SLP because despite 1 ATP is obtained by deactivation of propionyl-CoA, ATP was previously invested to produce the intermediates and for the CoA activations.

The different acidic character of the final products has an important effect on the energy requirements for their transport outwards the cell membrane into a medium that can have a different pH. These implications have been discussed in more detail before (see Chapter 2) and the particular role of formic acid, affecting the environmental conditions, will be discussed below.

With the objectives and the limitations of the existing MCF model defined and an extensive metabolic network available, the incorporation of more metabolic and physiological information is recommended for model improvement. In order to overcome the existing model limitations described above, several alternatives to increase the model capabilities appear as potentially interesting.

3.5. Metabolism and physiology in MCF modelling

Many of the model limitations identified correspond to metabolic and physiological uncertainties. Important issues to deal with, or to incorporate into, the MCF models are discussed below:

i) Incorporation of the roles of electron carriers other than NAD appears as a key issue for improving the model capacity to define the whole redox state of the system and the subsequent effects on the product spectrum. At this stage NAD is used just to close electron balances by assuming equilibrium with hydrogen. The incorporation of other electron carriers will add more definition to the system flexibility when the electron transfers between electron carriers are not possible or imply energy losses. Thus NADP, FAD, Fd could be incorporated, provided that the mechanisms of electron transfer from

one to other carrier are modelled and not just assumed in equilibrium. This will force to close redox balances for some carriers separately or to spend energy to transfer these electrons among them when possible. The study of the redox reactions between these carriers can provide the model with more information to predict much better the product spectrum and particularly the hydrogen production.

ii) Intracellular pH and homeostasis models can have major implications. The intracellular metabolite concentrations of acidic species are currently artificially limited by a maximum, to stay close to reality maintaining the cellular homeostasis. This limitation implies more energy costs to transport these species outwards from a cell at neutral pH into an acidic medium. The incorporation of an intracellular pH model would be crucial to avoid artificial limitation of the metabolite concentrations but also to permit intracellular pH variations, as occur in reality (Diez-Gonzalez and Russell, 1997), with the important effect on the ATP/ADP/P_i ratios (one proton is produced in the hydrolysis of ATP) and consequently on the whole energetics of the cell. This pH equilibrium model could incorporate more intracellular metabolites to achieve better accuracy. Such an intracellular pH model is not very complex from the structural perspective and for computing, fast numeric algebraic approximation methods exist.

iii) Formic acid as intermediate or final product. Formic acid is produced by the enzyme pyruvate formate lyase during the formation of acetyl-CoA from pyruvate and it is a highly acidic product. The potential importance of formic acid comes from its dual possibility of transport outwards as an acidic compound or of conversion into carbon dioxide and hydrogen (Sawers, 2005). The energetic implications of one or other possibility are different (see Figure 3.2).

If formic acid is not converted, its transport in acidic conditions is energetically expensive. The transport of formic acid outwards, into a more acidic environment, will require normally energy consumption and will decrease the biomass yield (see Chapter 2). On the other hand, if formic acid is converted to hydrogen and carbon dioxide, there is no need to transport the acidic compound outwards but its decomposition increases the carbon dioxide concentrations and the hydrogen partial pressure, causing eventually thermodynamic limitations of oxidative reactions.

Elevated hydrogen concentration will narrow the thermodynamic feasibility region for oxidations, like acetate formation, and requires lower intracellular concentration of acetic acid to proceed, with extra costs of transport of the acid as well. The role of carbon dioxide is also of importance in one of the pathways leading to propionate (see Figure 3.1) since it is involved in the formation of oxalacetate and decarboxylation of methylmalonyl-CoA by means of biotin.

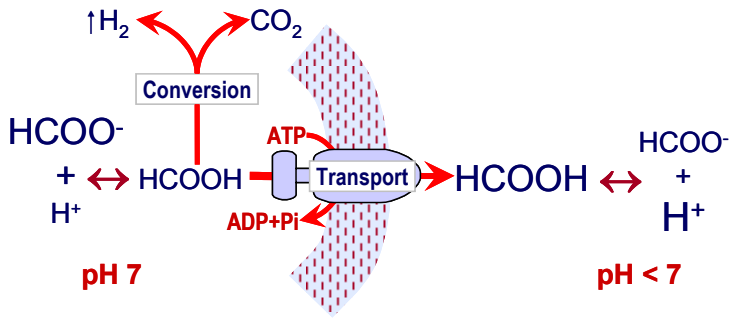

Figure 3.2. Formic acid conversion or transport outwards into an acid environment.

This makes formic acid conversion/transport a regulation mechanism of the cell interchanging energy costs associated with transport, with those related to intermediate concentrations in oxidation pathways. This has potential implications in the product spectrum obtained.

iv) Intracellular metabolite concentrations and in particular activated CoA species, if implementing a detailed network (see Figure 3.1). Some of the metabolite concentrations are estimated or simply taken from the literature but these values are of high importance for the energetics of the pathways. In particular reactions involving activations with CoA species are deeper studied below, seeking for eventual kinetic limitations at certain steps. These metabolite concentrations are directly linked with the cell homeostasis and with an eventual intracellular pH model. Measurement of intracellular metabolite levels will be vital for validation of MCF models.

v) The assumption of equilibrium between hydrogen and electron carriers must be validated experimentally since it has an enormous impact on the effect of the hydrogen partial pressure on the product spectrum. Measurement of intracellular metabolites will

provide more insight in the bioenergetic status of the electron transfer reactions. The assumption of equilibrium is furthermore related to the role of the different electron carriers, with separate redox cycles, and it is of major relevance for modelling hydrogen production in MCF.

vi) Incorporation of more metabolic pathways appears as interesting only accompanied with new products and with experimental methods that enable validation of the pathways proposed. Since the model is based on a bioenergetic approach, with optimum energy efficiency in the cell, the pathway to achieve the product does not affect much the energy obtained as long as the energy is conserved. For these reason the addition of more pathways leading to the same products seems not a priority at this stage.

Related to the metabolite levels, the effect of the hydrogen concentration on the equilibrium concentrations of intermediates, in particular CoA-species, is studied in more detail. High hydrogen levels can force very low concentrations of certain intermediates by thermodynamic restriction and cause a decrease in the flux through a pathway by kinetic limitation (Kleerebezem and Stams, 2000). The incorporation of kinetics for some processes is a necessary next step in MCF modelling, currently with a pure energetic approach.

3.6. Incorporation of kinetics into MCF models

Kinetic limited processes have been neglected in the existing MCF model. The model assumes a full bioenergetic control, with a given constant substrate uptake rate as input, and the only kinetic controlled process included is the free diffusion of undissociated acids through the cytoplasmic membrane. There are situations however, where the thermodynamic constraints can induce kinetic limitations, derived from the very low concentrations of intermediates. Apart from this, there are other processes that occur clearly far from thermodynamic equilibrium with limiting kinetics. This implies that kinetics can have an important effect on the MCFs systems and it must be addressed.

3.6.1. Thermodynamically induced kinetic limitations

Despite anaerobic metabolic reactions proceed close to thermodynamic equilibrium and make the thermodynamic control hypothesis defendable, there are situations where the thermodynamic constraints can also induce kinetic limitations.

Figure 3.3 presents the Gibbs free energy changes of the catabolic reactions from the extended metabolic network from Figure 3.1, in standard conditions at pH 7. The generation and consumption of ATP by SLP as well as the reduction/oxidation reactions by consumption/production of hydrogen are also indicated. Apart from glycolysis, there are three reactions that present high energy jumps. In the metabolism these reactions are known to produce energy by proton translocation and generation of proton motive force (Konings, 1985), that is converted into ATP by the ATPase system (see Chapter 2).

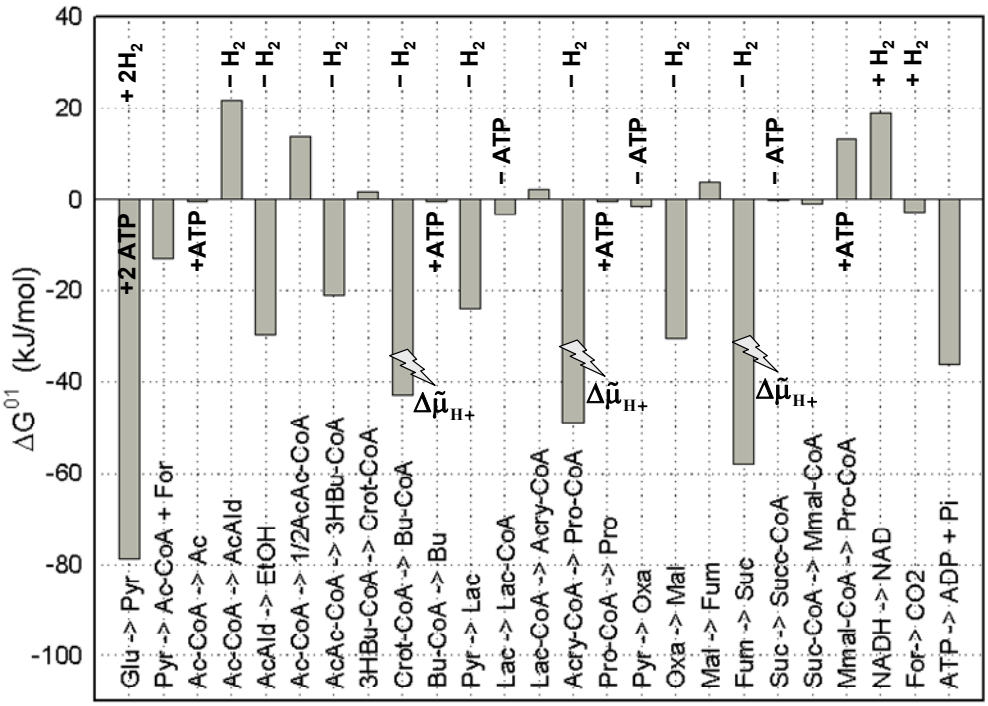

Figure 3.3. Gibbs free energy exchange of all the catabolic bioreactions from the extended metabolic network of MCF under standard conditions at pH 7.

The thermodynamics of the metabolic reactions define the intervals within which the intracellular metabolite concentrations must remain to enable the reactions to proceed. The most unfavourable reactions require higher substrates/products concentration ratios. This can eventually lead to extremely low and even unrealistic concentrations of some

metabolite within a series of reactions blocking the pathway. Despite no quantitative significant results could be attained at this stage yet, the effect has been investigated using estimated reasonable metabolite concentrations.

Figure 3.4 presents the results obtained when computing the equilibrium system of the extended metabolic network from Figure 3.1 (without the lactoyl-acryloyl-CoA route). The concentrations of the CoA metabolites are calculated for different partial pressures of hydrogen with a fixed total amount of CoA species in the cell. The computation consisted of the simultaneous solution of the equilibrium system and the mass balance of CoA species by iteration with the free CoASH concentration until convergence.

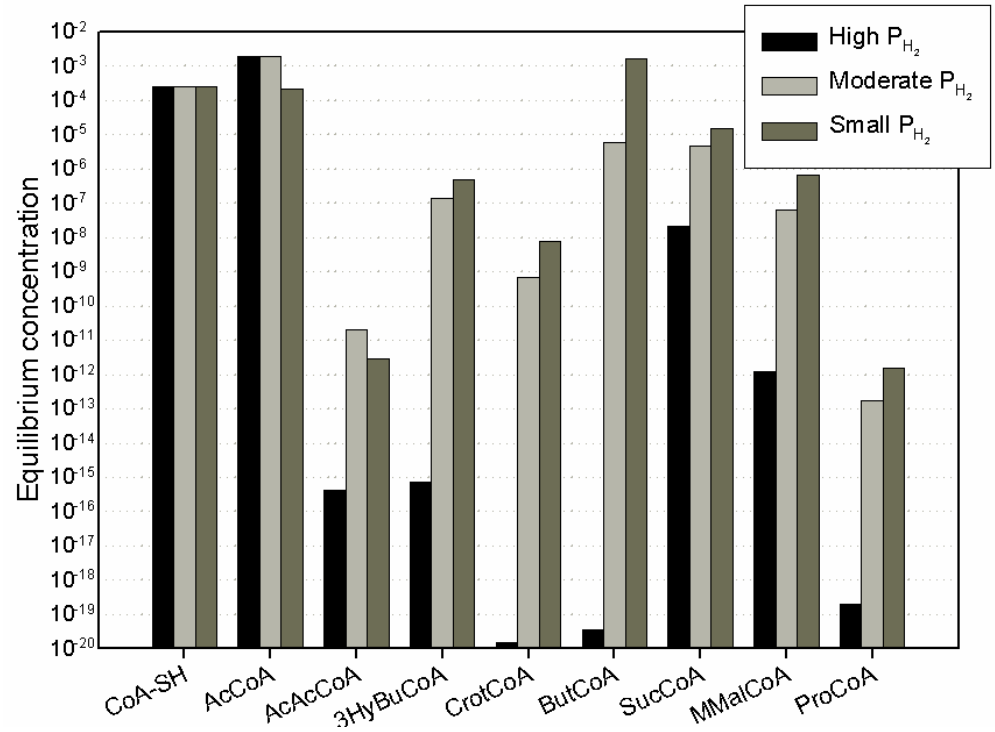

Figure 3.4. Illustration of the effect of the hydrogen concentration on the equilibrium concentrations of CoA species in a metabolic network similar to Figure 3.1.

Despite the unrealistic concentrations values shown in Figure 3.4 they illustrate the important changes in concentrations that thermodynamic constraints can cause, leading to extremely small values, with impossible further conversion due to kinetic limitation. The modelling of these phenomena from a thermodynamic perspective consists of a shift function (feasible/unfeasible) on the reaction flux. From a kinetic perspective however it could be implemented as a transition from zero order to higher order kinetics. Thus, for certain situations, a transition to concentration dependent fluxes should be incorporated to address the transitions from feasible to unfeasible thermodynamic sceneries.

Lumped thermodynamic approaches neglect the fact that, for an intermediate reaction within a series, the concentration of a certain metabolite can become extremely low and therefore its further conversion kinetically impossible. Some tentatives have been reported dealing with this issue (Westerhoff, 1982) and, from the metabolic engineering field, there are kinetic models available dealing with reactions through minimum driving forces (Visser and Heijnen, 2003).

3.6.2. Transport kinetics of substrates and products

In MCF systems, under certain conditions, it can occur that the limiting step changes from a transport process to a different one, corresponding to the uptake of other substrate or to the excretion of a product. This may happen if low substrate or high product concentrations occur. Incorporation of kinetics of substrates and products transport should permit to reproduce these situations.

Such phenomena could imply that the optimum performance of the microbial population, given by selective pressure, changes from a maximum yield to a maximum growth rate criterion for example. Since this would change deeply the model principles it must be properly addressed as well.

In addition to this, the use of proper kinetics for transport processes enables the application of the model to MCF systems with several substrates together. A wider range of sceneries appear thus, where the culture can convert a mixture of substrates into other products, by efficiently harvesting the energy associated to the conversions.

3.7. Mass transfer processes

Mass transfer limitations from liquid to gas have been identified as of high importance and particularly the limitation in the transfer rates occurring for hydrogen and carbon dioxide. Such phenomena are relatively easy to implement and appear as very important. The steady state liquid concentrations are highly dependent on the transfer rates and when these are slow, liquid concentrations are far from equilibrium. This has an immediate effect on the thermodynamics of the system. For example, a supersaturation of hydrogen in the liquid, due to slow transfer to the gas phase, causes that the effective concentration experienced by the microorganisms is much higher than the equilibrium partial pressure in the gas, with the subsequent thermodynamic implications. Other mass transfer limitations exist also between the microorganisms and the liquid medium and could be considered as well by defining the effective concentrations experienced by the microbes.

3.8. Computational improvement of MCF models

Currently the computation of the MCF model implies the solution of a global optimisation problem. The complexity of the global, heavily constrained, optimisation problem of several variables made necessary the utilisation of a certain level of heuristics in the optimisation algorithm to obtain reliable results in reasonable computing times.

Several options can be explored for improving the computing efficiency:

i) Enhanced heuristics, by incorporating more knowledge of the system to facilitate the finding of the global optimum and faster discard of unfeasible sceneries.

ii) Linearisation of the system by approximation of the logarithmic thermodynamic systems for faster reach of the global optimum.

iii) Model reductions for suboptimisation. Some parts of the models could be temporary neglected during the optimisation to faster find a suboptimum, which will be used as starting point for the global optimisation problem with the complete model.

3.9. Route plan for MCF modelling

Considering the results obtained with the MCF model developed (Rodríguez *et al.*, 2006), the analysis of the several alternatives for model improvements and, in view of the objectives and major limitations, a route plan for modelling MCF is proposed.

1. Mass transfer kinetics from liquid to gas phase. This is relatively easy to implement and has an immediate quantitative impact on the effective concentrations (particularly hydrogen) experienced by the conversion system (the mixed culture).

2. Formic acid regulated conversion/transport mechanism. This appears as a potentially very important regulation mechanism for microorganisms that permits to exchange energy associated with active transport (very pH dependent) and with the thermodynamics of the bioconversions (very hydrogen dependent). Experimental data (Temudo, 2005) have shown formic acid as an important product under particular conditions and therefore its incorporation appears as necessary as another fermentation product.

3. Alternative electron carriers. This is a necessary step and, if the mechanisms of electron transfer between carriers are well implemented and present energy losses or constrained transfers, major implications are expected for the product spectrum.

4. Kinetics of transport processes. This would extend the model applicability to multisubstrate cases and be able to deal with situations where the limiting step changes to other than a given substrate uptake rate.

5. Thermodynamics induced kinetic limitations. The cases where pathways are blocked by extremely low intermediate concentrations (e.g. affecting the CoA species) can explain the occurrence of some products under certain conditions (e.g. the case of lactate production when the CoA pathways are saturated).

6. Intracellular pH and homeostasis model. This will be a big step towards a very detailed definition of the microbial system. Intracellular pH changes, already reported in the literature, would be possible and it has important implications for reversible transport of products as substrates.

7. Segregation to multiple microbial species. If the fundamental differences among microbial species are identified, the possibility of segregation of the lumped mixed microbial population into several microorganisms could be proposed. The consideration of several microbial species, with different metabolic networks, could be the platform on which to incorporate ecological aspects and other selective pressures. Consideration of several microbial species constrains the system additionally, by forcing to close mass balances and thermodynamic laws separately for each microorganism.

This tentative route plan requires extensive experimental information in order to validate each step and to avoid unnecessary model upgrades that add more complexity, without supplying realistic insight in the process. The priorities within the route plan proposed respond to the expected contribution to model improvement and to the difficulty of implementation or identification as well as level of uncertainty. The wide range of phenomena to be addressed to model these processes clearly encourages for cooperative and multidisciplinary research.

Microbiology knowledge can contribute largely to the revision of the fundamental hypotheses used in MCF modelling up to now. The current approach is based in a growth based selective pressure but other selective pressures are also possible, They could be relevant to the key regulation mechanisms of product formation and redefine the objective function of the system optimisation. Pure microbial cultures could be also useful to investigate the effects of environmental variables like pH or hydrogen concentration with a lower number uncertainties present in the study.

An interesting research area emerges from this modelling approach for MCFs with the objective of developing more sustainable technologies to recover valuable products from wastes.

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Chapter 4

EXTENSION OF THE ANAEROBIC DIGESTION MODEL NO.1 FOR ETHANOL DEGRADATION

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Chapter 4

EXTENSION OF THE ANAEROBIC DIGESTION MODEL NO.1 FOR ETHANOL DEGRADATION

Contents

Abstract

In this chapter the applicability of the IWA Anaerobic Digestion Model No.1 (ADM1) is extended to anaerobic digestion of ethanolic wastewater. A structural modification of the model, incorporating the conversion process of ethanol degradation, is proposed. For this purpose, a model implementation in *Matlab/Simulink* is previously developed and validated by comparison with implementations in other platforms. Based on this platform the modified model is implemented and applied to simulation of overload experiments in a pilot scale UASB-AF reactor treating diluted wine.

4.0. Summary

The importance of ethanol degradation during anaerobic treatment of wastewaters from wineries motivates for the study of the process, aiming at its incorporation to the IWA Anaerobic Digestion Model No.1 (ADM1).

The ADM1 is first implemented on a *Matlab/Simulink* platform and the results are compared with those obtained with other platforms as *West* and *Aquasim*. Computational issues derived from the study of the different platforms are also discussed, regarding especially the computing bottlenecks during simulations.

To extend the ADM1 for ethanol treatment, several experiments are conducted in a pilot scale anaerobic reactor consisting of a hybrid UASB-AF of about 1.2 m^3, treating diluted wine. The experiments are used to identify the kinetic and stoichiometric parameters of the ethanol conversion into volatile fatty acids (VFAs), aiming at their incorporation in the model. A description of the pilot plant used is presented as well as of the architecture of the on-line monitoring and control system used.

The structure of the ADM1 is modified to incorporate ethanol degradation process and using the kinetic and stoichiometric parameters, experimentally estimated, for ethanol and its catabolic products. The modified ADM1 developed is applied to simulate overload experiments in the same pilot scale UASB-AF reactor. In view of the results obtained, the applicability of the modified model under this approach is discussed. The model application is recommended mainly for dynamic situations as design, tuning and virtual testing of controllers, definition of start-up protocols, etc. More mechanistically correct models require a variable stoichiometry, as it has been discussed in previous chapters of this thesis. This must be kept in mind when defining the scope of ADM1 based models.

4.1. Introduction

Anaerobic digestion processes consist of a set of conversions of organic matter into methane and carbon dioxide, occurring in absence of oxygen (Lettinga, 1995; Lema *et al.*, 1992). Depending on the substrate characteristics, the number of steps required to achieve the final products varies. The successive conversions from substrate to products are carried out by different groups of microorganisms (Stams, 1994). Thus, the products of a certain microbial conversion are the substrates for the subsequent microbial conversion until the final monocarbonated products (CH_4 and CO_2).

In order to assist design and operation of anaerobic wastewater treatment plants, several models have been developed in the last decades (McCarty and Mosey, 1991; Angelidaki *et al.*, 1993; Siegrist *et al.*, 1993; Vavilin *et al.*, 1994; von Munch *et al.*, 1999; Batstone *et al.*, 2000). Some models dealt mainly with more specific substrates or certain operation conditions with different levels of complexity.

Recently, a generic process model has been developed by the IWA task group for mathematical modelling of anaerobic digestion processes, resulting in the Anaerobic Digestion Model No.1 (ADM1) (Batstone *et al.*, 2002). The ADM1 was created with the intention of being useful-for-general rather than accurate-for-specific applications. Thus, the model structure was confined to the most relevant processes for general applications and neglected some others, more important for specific cases.

One important application of anaerobic digestion is the treatment of wastewater from breweries and wine industries. Anaerobic treatment of this type of wastewater has been investigated to incorporate control strategies to improve stability and robustness of the process (Bernard *et al.*, 2005; Puñal *et al.*, 2002; Rodríguez *et al.*, 2005b; Ruiz *et al.*, 2005). Wastewater from wine factories and breweries contains ethanol as the main source of COD that must be first converted to VFAs and hydrogen for subsequent methanisation.

In this work, the ADM1 applicability is extended to ethanolic wastewater treatment by incorporating a model modification dealing with the ethanol degradation process. Experiments in a pilot scale anaerobic reactor will provide information for the structural modifications as well as parameter values.

4.2. The IWA Anaerobic Digestion Model No.1

A few years ago the IWA task group of mathematical modelling of anaerobic digestion initiated the development of a general purpose model for anaerobic digestion processes. The motivations for developing such a generic model were to seek for: (i) an increased model application for design, operation and optimisation; (ii) a common basis for further model developments and validation with comparable results; (iii) an improvement in the technology transfer to industry (Batstone *et al.*, 2002). The resulting ADM1 is widely used today as a reference model for research and design purposes. Many applications and developments based on the ADM1 have been recently reported (Batstone *et al.*, 2005).

The ADM1 considers three types of processes: (i) the biochemical conversions that are carried out by microorganisms degrading stepwise solid and soluble substrates to methane and carbon dioxide. Decay of biomass and hydrolysis of particulates are included among these biochemical conversions; (ii) the physicochemical processes consisting of the acid-base equilibria (ion dissociations); (iii) the mass transfer processes between liquid and gas phase.

Figure 4.1 presents schematically the conversions and species considered in the ADM1 while the stoichiometries of all the conversion processes are shown in Figure 4.2a-b. The ADM1 assumes constant stoichiometry, independent from the environmental conditions, and the observed changes in the state variables are controlled by the kinetics of the conversion processes. Table 4.1 shows the kinetic expressions of the biochemical conversions. First order kinetics are considered for disintegration, hydrolysis and decay processes, while Monod-type expressions are used for the rest of bioconversions, including terms that modify the reaction rates as a function of the concentration of inhibitors and/or of environmental conditions.

The physicochemical conversions can be implemented either by modelling the acid-base equilibria as fast kinetic processes approaching thermodynamic equilibrium (DE model implementation), or by solving the chemical equilibrium in a separate procedure as more simple algebraic expressions (DAE model implementation).

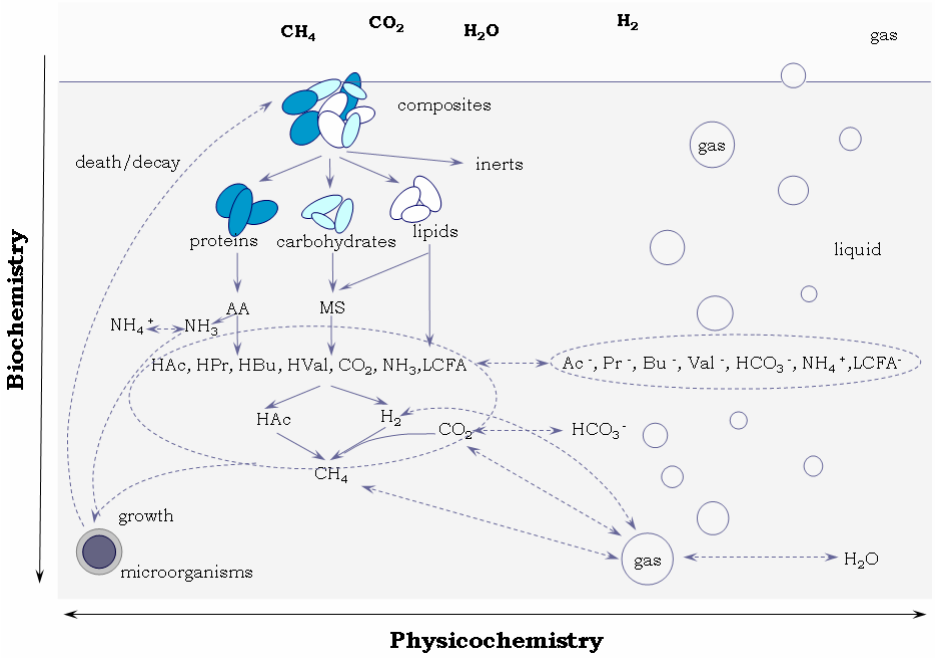

Figure 4.1. Biochemical and physicochemical processes considered in the ADM1. (adapted from A. Puñal with permission)

Since the publication of the scientific report describing the ADM1 (Batstone *et al.*, 2002) the model has been extensively used in both academic and practical applications. Limitations of the model have been also identified and extensions for specific applications have been proposed (Batstone *et al.*, 2005). Among the published applications, many of them dealt with mixed tank systems including mixed sludge digesters, manure digesters and even UASB systems. A new COST benchmark for wastewater treatment plants has been proposed incorporating anaerobic digestion by means of the ADM1 (Jeppson *et al.*, 2005). The EU Project *TELEMAC* (Bernard *et al.*, 2005), dealing with remote monitoring, advanced control and operation of anaerobic wastewater treatment plants, applied extensively the ADM1 in many stages of the project. Among the applications, the ADM1 was used as a virtual plant to evaluate different control strategies and software sensors for anaerobic digesters (Bernard *et al.*, 2005). A model-based training tool was also developed based on the ADM1 since this model can predict the behaviour of the system during overloads complex behaviour, required for advanced operator training purposes.

	Component →	1	2	3	4	5	6	7	8	9	10	11	12
	Process ↓	Ssu	Saa	Sfa	Sva	Sbu	Spro	Sac	Sh2	Sch4	Sic	Sin	Si
1	Disintegration											inN	fsi_xc
2	Hydrolysis of Carbohydrates	1											
3	Hydrolysis of Proteins		1										
4	Hydrolysis of Lipids	1-ffa_li		ffa_li									
5	Uptake of Sugars	-1				(1-Ysu)·fbu_su	(1-Ysu)·fpro_su	(1-Ysu)·fac_su	(1-Ysu)·fh2_su		$\nu_{ic\ 6}$	-Ysu·Nbac	
6	Uptake of Amino Acids		-1		(1-Yaa)·fva_aa	(1-Yaa)·fbu_aa	(1-Yaa)·fpro_aa	(1-Yaa)·fac_aa	(1-Yaa)·fh2_aa		$\nu_{ic\ 7}$	Naa-Yaa·Nbac	
7	Uptake of LCFA			-1				(1-Yfa)·0.7	(1-Yfa)·0.3			-Yfa·Nbac	
8	Uptake of Valerate				-1		(1-Yc4)·0.54	(1-Yc4)·0.31	(1-Yc4)·0.15			-Yc4·Nbac	
9	Uptake of Butyrate					-1		(1-Yc4)·0.8	(1-Yc4)·0.2			-Yc4·Nbac	
10	Uptake of Propionate						-1	(1-Ypro)·0.57	(1-Ypro)·0.43		$\nu_{ic\ 11}$	-Ypro·Nbac	
11	Uptake of Acetate							-1		(1-Yac)	$\nu_{ic\ 12}$	-Yac·Nbac	
12	Uptake of Hydrogen								-1	(1-Yh2)	$\nu_{ic\ 13}$	-Yh2·Nbac	
13	Decay of Biomass Xsu												
14	Decay of Biomass Xaa												
15	Decay of Biomass Xfa												
16	Decay of Biomass Xc4												
17	Decay of Biomass Xpro												
18	Decay of Biomass Xac												
19	Decay of Biomass Xh2												
		Monosaccharides (kg COD/m^3)	Amino Acids (kg COD/m^3)	Long Chain Fatty Acids (kg COD/m^3)	Total Valerate (kg COD/m^3)	Total Butyrate (kg COD/m^3)	Total Propionate (kg COD/m^3)	Total Acetate (kg COD/m^3)	Hydrogen (kg COD/m^3)	Methane (kg COD/m^3)	Inorganic Carbon (kmol-C/m^3)	Inorganic Nitrogen (kmol-N/m^3)	Soluble Inerts (kg COD/m^3)

Figure 4.2a. Stoichiometry matrix of the ADM1 for the soluble species.

	Component →	13	14	15	16	17	18	19	20	21	22	23	24
	Process ↓	Xc	Xch	Xpr	Xli	Xsu	Xaa	Xfa	Xc4	Xpro	Xac	Xh2	Xi
1	Disintegration	-1	fch_xc	fpr_xc	fli_xc								fxi_xc
2	Hydrolysis of Carbohydrates		-1										
3	Hydrolysis of Proteins			-1									
4	Hydrolysis of Lipids				-1								
5	Uptake of Sugars					Ysu							
6	Uptake of Amino Acids						Yaa						
7	Uptake of LCFA							Yfa					
8	Uptake of Valerate								Yc4				
9	Uptake of Butyrate								Yc4				
10	Uptake of Propionate									Ypro			
11	Uptake of Acetate										Yac		
12	Uptake of Hydrogen											Yh2	
13	Decay of Biomass Xsu	1				-1							
14	Decay of Biomass Xaa	1					-1						
15	Decay of Biomass Xfa	1						-1					
16	Decay of Biomass Xc4	1							-1				
17	Decay of Biomass Xpro	1								-1			
18	Decay of Biomass Xac	1									-1		
19	Decay of Biomass Xh2	1										-1	
		Composites (kg COD/m^3)	Carbohydrates (kg COD/m^3)	Proteins (kg COD/m^3)	Lipids (kg COD/m^3)	Sugar Degraders (kg COD/m^3)	Aminoacid Degraders (kg COD/m^3)	LCFA Degraders (kg COD/m^3)	Vale- & Butyrate Degraders (kg COD/m^3)	Propionate Degraders (kg COD/m^3)	Acetate Degraders (kg COD/m^3)	Hydrogen Degraders (kg COD/m^3)	Particulate Inerts (kg COD/m^3)

Figure 4.2b. Stoichiometry matrix of the ADM1 for the particulate species.

Table 4.1. Kinetic expressions of the bioconversions in ADM1.

Process name	Conversion rate (kgCOD/m^3·d)
1. Disintegration	$k_{dis} \cdot X_c$
2. Hydrolysis of carbohydrates	$k_{hyd\_ch} \cdot X_{ch}$
3. Hydrolysis of proteins	$k_{hyd\_pr} \cdot X_{pr}$
4. Hydrolysis of lipids	$k_{hyd\_li} \cdot X_{li}$
5. Uptake of sugars	$k_{m\_su} \cdot \frac{S_{su}}{K_{s\_su} + S_{su}} \cdot I_{in\_lim} \cdot I_{ph} \cdot X_{su}$
6. Uptake of aminoacids	$k_{m\_aa} \cdot \frac{S_{aa}}{K_{s\_aa} + S_{aa}} \cdot I_{in\_lim} \cdot I_{ph} \cdot X_{aa}$
7. Uptake of LCFA	$k_{m\_fa} \cdot \frac{S_{fa}}{K_{s\_fa} + S_{fa}} \cdot I_{in\_lim} \cdot I_{ph} \cdot I_{h2\_fa} \cdot X_{fa}$
8. Uptake of valerate	$k_{m\_c4} \cdot \frac{S_{va}}{K_{s\_va} + S_{va}} \cdot I_{in\_lim} \cdot I_{ph} \cdot I_{h2\_c4} \cdot I_{bu} \cdot X_{c4}$
9. Uptake of butyrate	$k_{m\_c4} \cdot \frac{S_{bu}}{K_{s\_bu} + S_{bu}} \cdot I_{in\_lim} \cdot I_{ph} \cdot I_{h2\_c4} \cdot I_{va} \cdot X_{c4}$
10. Uptake of propionate	$k_{m\_pro} \cdot \frac{S_{pro}}{K_{s\_pro} + S_{pro}} \cdot I_{in\_lim} \cdot I_{ph} \cdot I_{h2\_pro} \cdot X_{pro}$
11. Uptake of acetate	$k_{m\_ac} \cdot \frac{S_{ac}}{K_{s\_ac} + S_{ac}} \cdot I_{in\_lim} \cdot I_{ph\_ac} \cdot I_{nh3\_xac} \cdot X_{ac}$
12. Uptake of hydrogen	$k_{m\_h2} \cdot \frac{S_{h2}}{K_{s\_h2} + S_{h2}} \cdot I_{in\_lim} \cdot I_{ph\_h2} \cdot X_{h2}$
13. Decay of sugar consumers	$k_{dec} \cdot X_{su}$
14. Decay of aminoacid consumers	$k_{dec} \cdot X_{aa}$
15. Decay of LCFA consumers	$k_{dec} \cdot X_{fa}$
16. Decay of C4 consumers	$k_{dec} \cdot X_{c4}$
17. Decay of propionate consumers	$k_{dec} \cdot X_{pro}$
18. Decay of acetate consumers	$k_{dec} \cdot X_{ac}$
19. Decay of hydrogen consumers	$k_{dec} \cdot X_{h2}$

Much of the criticism about the ADM1 focus on the complexity of the model. Sometimes the model is considered as too simple for particular applications although that simplicity can be overcome by adding extensions addressing the requirements of the specific application. On the other hand there is also criticism based on the high complexity of the model for more general purposes although the ADM1 can be used as the basis for more simple models. The issue of model reduction -decreasing parameter estimation requirements and the time required for implementation and simulation- is addressed in Chapter 6 of this thesis where a model reduction technique using statistical tools is proposed.

The model stoichiometry based only in catabolic reactions was also identified as an important limitation of the ADM1. This causes that, in cases where carbon limitation occurs, biomass growth takes the carbon from the inorganic form, unrealistically for most organisms (Kleerebezem and van Loosdrecht, 2004). Despite the impact of this is rather limited because of the small biomass yields in anaerobic digestion, it was identified as a correction to be incorporated (Batstone *et al.*, 2005).

Several extensions of the model for specific applications have been demanded and, since the ADM1 is easily extendible, incorporations of additional processes have been developed. Among the most requested extensions, sulphate reduction was a rather complex one (Fedorovich *et al.*, 2003) considering that sulphate is not only an electron acceptor for oxidation of VFAs but also that it has a high affinity for molecular hydrogen. For an integrated balance of phosphorous in wastewater treatment plants, an extension of the ADM1 to phosphorous cycle has been demanded and despite it would be relatively simple, it has not been considered yet. The same applies for salts precipitation that can be introduced either as equilibrium or as more complex reactions (Batstone *et al.*, 2005). Other specific extensions of the model like ethanol degradation, considering only a direct transformation into acetate, have been reported (Batstone *et al.*, 2004).

From the main lacks of the ADM1 identified during the last years namely: (i) regulation of products from glucose fermentation; (ii) proper values and variability studies of parameters; and (iii) extensions for specific processes that need to be faced, the last two items have been reasonably addressed in the literature but the key limitation of the regulation of glucose fermentation products remains uncovered.

It is known that environmental factors as pH or hydrogen concentration affect the stoichiometry of product formation in carbohydrate fermentations (Mosey, 1983; Costello *et al.*, 1991). The constant stoichiometry parameters used for glucose fermentation in ADM1 are not representative of the acidogenesis process. The increasing interest in non methanogenic carbohydrate fermentation technologies makes relatively urgent to develop better models of these processes. The work presented previously (see Chapter 2 and Chapter 3) contributes to fill this gap in the modelling of product formation from carbohydrate MCFs (Rodríguez *et al.*, 2006) and the potential of integrating this advances in the ADM1 are assessed in Chapter 5.

4.3. Implementation of the ADM1 in *Matlab/Simulink*

The ADM1 with algebraic acid-base equilibrium is implemented in *Matlab/Simulink*, chosen as the most adequate platform due to, among other characteristics, its flexibility for further structural modifications. Using *Matlab/Simulink* allows the straightforward integration of the model with other blocks containing other elements (e.g. controllers) and facilitates its application on advanced controllers development, tuning and testing by just connecting *Simulink* blocks (Rodríguez *et al.*, 2005b; Mailleret *et al.*, 2004).

The ADM1 is implemented using *Matlab S-Functions* since they are very suitable to build customised and very flexible blocks. Separate blocks are created for the reactor mass balances, the kinetics of the conversion processes and the computation of the stoichiometry matrix. Figure 4.3 shows the resulting block diagram of the ADM1 implementation. The physicochemical processes are assumed in equilibrium adopting therefore the DAE model implementation.

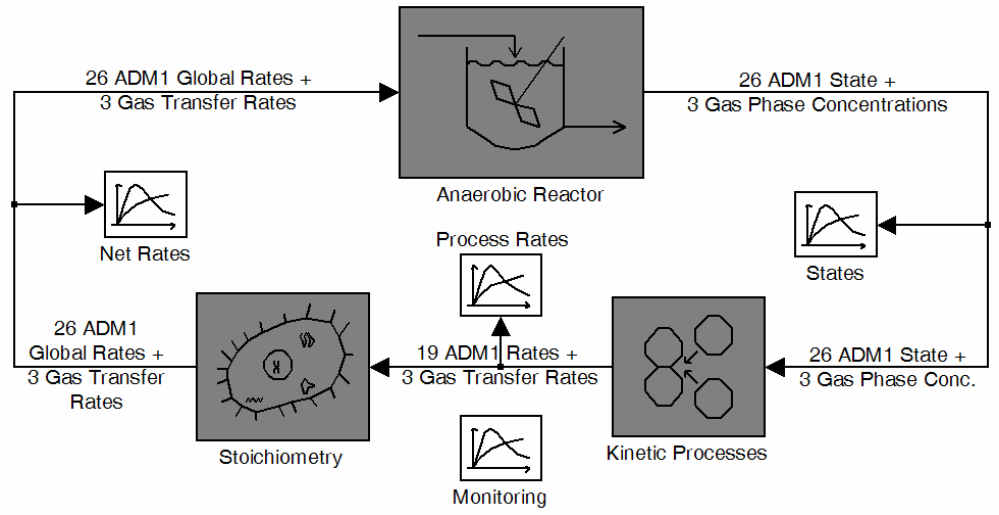

Figure 4.3. *Simulink* blocks diagram of the ADM1 implementation developed.

The reactor model is implemented in a separate *S-Function* file (*ad_reactor.m*) where the corresponding mass balances of the liquid, solid and gas species are computed. This modularity allows different reactor models (particularly for modified biomass retention systems) to be incorporated. Finally an additional solid retention time is applied to the mass balances of the solid species according to the ADM1 scientific and technical report (Batstone *et al.*, 2002)

The kinetics of all conversion processes (Table 4.1), including their inhibition terms, are implemented as an *S-Function* in the file *adm1_kinetics.m* and the physicochemical calculations in the file *adm1_physchem.m*. The physicochemical module includes a function that solves the charge equation using a Newton-Raphson algorithm and calculates the current reactor pH from the concentrations of the ionic species.

By multiplying the stoichiometry matrix (see Figure 4.2a-b) by the conversion rates, the net generation term for each species, to be used in the mass balance differential equation, is obtained. This calculation is also implemented as an *S-Function* in the file *adm1_stoichiometry.m*.

Finally, to generate the input to the system in terms of feeding composition and flow rate, a specific *Matlab S-Function* was built-up (named *ad_feeding.m*). By providing an influent characterisation matrix with feed flow rate and species concentrations versus time, all experiments involving any influent changes in terms of flow and composition can be simulated. The function is flexible to leave any influent characteristic (e.g. the flow rate) open to be on-line given by other block (e.g. a controller).

4.3.1. Validation of the *Matlab/Simulink* implementation

In order to validate the model implementation in *Matlab/Simulink* a comparison with two other ADM1 implementations in other platforms, including *West* and *Aquasim*, is performed. Firstly, the steady state output of the *Matlab/Simulink* implementation is compared with the COST Benchmark simulation (Rosen and Jeppson, 2002) and with a *West* implementation (Zaher *et al.*, 2003). Figure 4.4 shows the variables that presented some differences between *Matlab/Simulink* and COST/*West* results, the rest of state variables showing relative differences smaller than 0.01%. The very small differences suggest that they are not due to programming errors. The implementation is therefore considered validated for the steady state output.

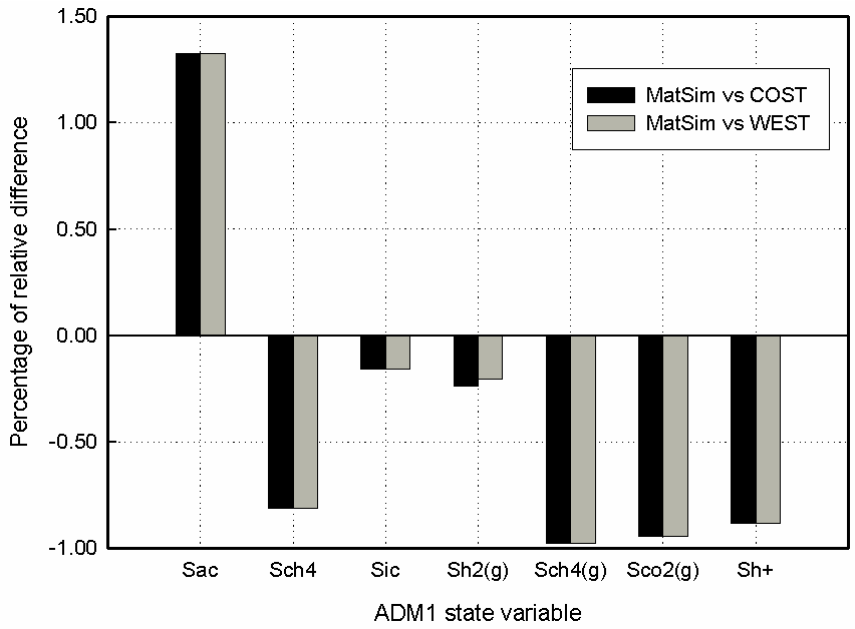

Figure 4.4. Differences found in some steady state values between the *Matlab/Simulink* implementation and the COST and *West* implementations.

Experimental data from a lab scale CSTR with a 2 L liquid volume are used (Zaher *et al.*, 2003) to validate dynamically the *Matlab/Simulink* implementation by comparison with the *West* and *Aquasim* implementations. The reactor was fed by charges of fresh wastewater in a semi-continuous mode. The wastewater, collected from an alcoholic distillery plant, contained high concentration of nitrogen mainly in the form of protein. The composition of the wastewater is shown in Table 4.2 and due to its high concentration it was diluted before feeding to the reactor. The complexity of the wastewater, containing probably several unmeasured compounds with possible slight toxic effects for some microbial populations, introduced an extra source of uncertainties in the experimental results. The initial inoculum of the reactor (6 gVSS/L) was obtained from the pilot-scale UASB-AF reactor described below after a period treating dextrose rich water.

Figure 4.5 and Figure 4.6 show the simulation results obtained with the three implementations of ADM1, using the same parameters, for the same input. As it can be observed the three implementations provide very similar results and therefore the implementation in *Matlab/Simulink* can be considered fully validated.

Table 4.2. Composition of the wastewater from the alcoholic distillery plant.

Magnitude	Value	Units
Total COD	75	g/L
Soluble COD	71	g/L
TOC	29.2	g/L
COD/TOC	2.6	g/g
Acetic acid	6.39	g/L
Propionic acid	0.07	g/L
n-Butiric acid	0.47	g/L
TSS	1.7	g/L
VSS	1.63	g/L
Protein	4	g/L
$N\text{-}NH_4^+$	126	ppm
NO_X	0	ppm
SO_4^{2-}	709	ppm
PO_4^{3-}	1142	ppm
Sugars	6.4	g/L

Apart from the validation of the model implementation, the results obtained from the application of the ADM1 to the experiment in the CSTR, treating diluted industrial wastewater, evidence some limitations. The most important problem is the influent characterisation, the wastewater composition was estimated from average concentrations of the industrial wastewater (see Table 4.2) derived from total COD, soluble COD, TOC, acetate, propionate, butyrate, TSS, VSS, protein, sugars, ammonia and sulphates. When dealing with complex wastewater, the information available for a proper influent characterisation is normally insufficient and causes a handicapped performance of the model. Despite there are useful methodologies available to make the influent characterisation more straightforward (Kleerebezem and van Loosdrecht, 2005), it remains a major limitation in the ADM1 applicability.

Even in absence of a good characterisation of the influent, the tempting practice of making small adjustments in influent characteristics to improve the fitting of the model to the experimental data is not recommended. This introduces wrong information in the system and the results obtained are meaningless for parameter identification purposes as well as of questionable reproducibility. The same data set should not be used for dual purpose of parameter estimation and influent characterisation.

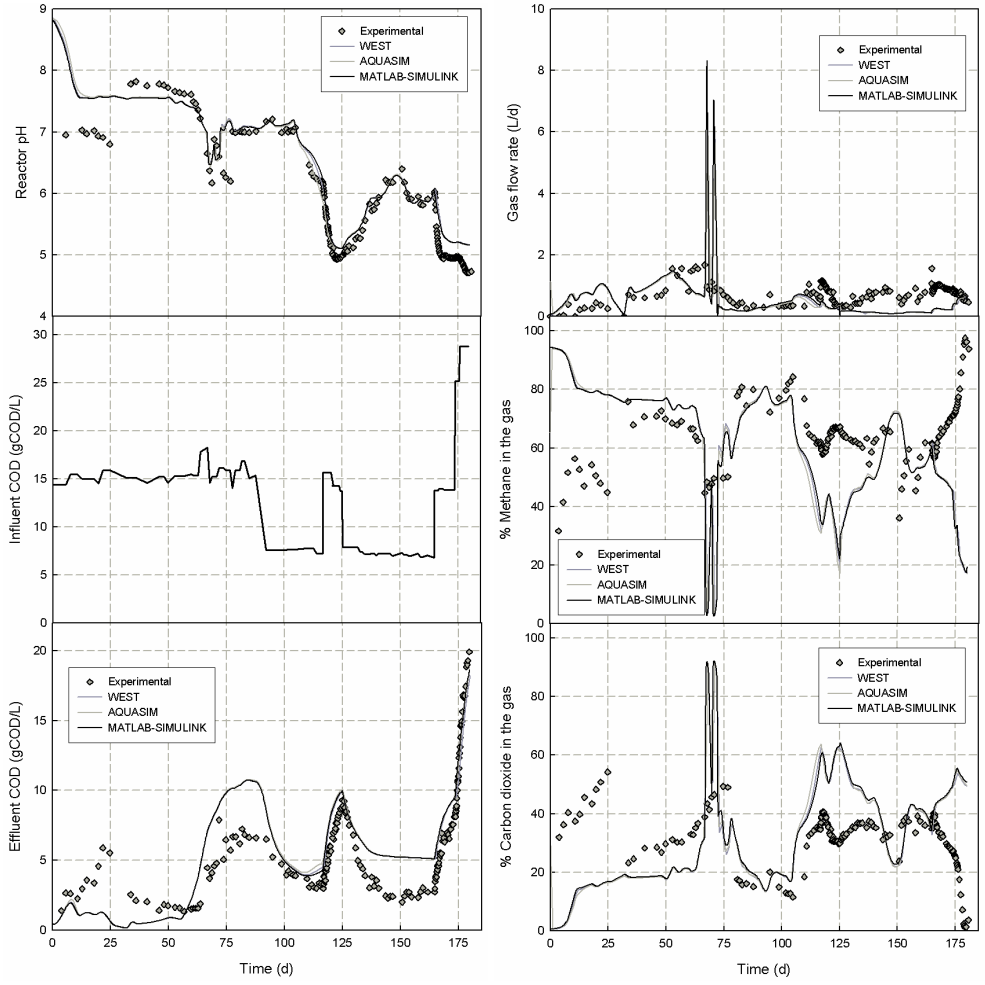

Figure 4.5. Simulation results of the *Matlab/Simulink*, *West* and *Aquasim* implementations of the ADM1 for the experiment in the 2 L CSTR reactor (COD, pH and gas composition).

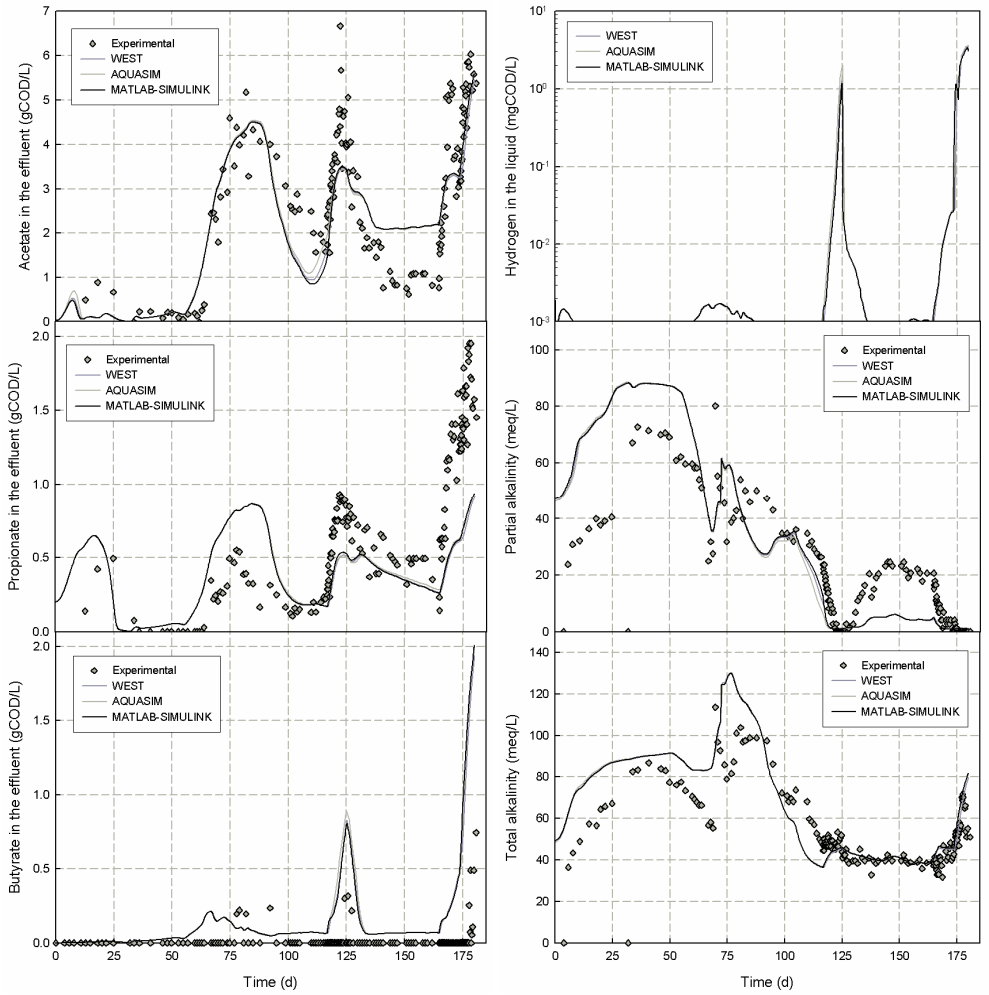

Figure 4.6. Simulation results of the *Matlab/Simulink*, *West* and *Aquasim* implementations of the ADM1 for the experiment in the 2 L CSTR reactor (VFAs, hydrogen and alkalinity).

The limited correspondence between experimental data and the ADM1 shown in Figure 4.5 and Figure 4.6 evidence the model limitations when a poor influent characterisation is available. The ADM1 could predict qualitatively the experimental data but a model reduction technique would be valuable to permit the use of the model with a less detailed and more accurate influent characterisation.

4.3.2. Computational issues

The wide range of time constants existing in the ADM1 from months to milliseconds (e.g. for biomass growth and for acid-base equilibrium) causes limitations in the simulation performance, given by the error tolerance during the integration of the fast changing states. These different time scales imply that, for the slow changing states, the fast states could be considered, from a practical point of view, as changing instantaneously. Thus, these fast changing states could be described by algebraic instead of differential equations, without major errors, with the subsequent reduction in the computing requirements for integration of state variables (Rosen *et al.*, 2005).

In the scientific and technical report of the ADM1 (Batstone *et al.*, 2002) it is suggested that the acid-base equilibrium and pH can be calculated by algebraic equations. In the *Matlab/Simulink* implementation, the calculation of the pH is performed by solving the charge balance using the Newton-Raphson algorithm on an implicit algebraic equation (Batstone *et al.*, 2002).

During the simulation of several case studies, including the case from the previous section, two major bottlenecks demanding computing time are identified: (i) The numeric pH calculation; and (ii) the integration of the hydrogen mass balance due to the high stiffness of the hydrogen concentration. During several applications of the implementation developed, the convenience of converting the differential equation of the hydrogen concentration state variable into an algebraic approximation is recognised. This transformation was not carried out here because the system performance is acceptable for the intended purposes but it was later confirmed and implemented by other authors (Rosen *et al.*, 2005).

Improving the computing requirements of the ADM1 simulations is of major importance when dealing with distributed parameter applications (e.g. biofilm modelling) as well as to conduct sensitivity analysis or other procedures demanding repeated simulation. Substitution of differential equations by algebraic for pH and hydrogen concentration is very recommendable in terms of error assumed versus simulation speed.

4.4. Pilot plant experiments with ethanol

4.4.1. Description of the pilot plant

The pilot plant used for the anaerobic treatment of ethanolic wastewater consists of a hybrid UASB-UAF reactor of about 1.2 m^3 of useful volume. The UASB zone is in the bottom part (about 75% of the total volume) while the anaerobic filter, consisting of rings of 50 mm long and diameter, is on the top of the reactor supported by a metallic grid. Finally the head space of the reactor is of 0.8 m^3 and everything is jacketed by thermal isolating material to avoid heat loss (Ruiz, 2005).

The wastewater used is synthetic and consists of diluted wine prepared *in-situ*. The wine and the dilution water are pumped independently so that the concentration can be controlled by changing the flows ratio. Another pump controls the nutrients and alkalinity dosage. Finally a recycling pump is responsible for both the mixing level desired in the reactor and the temperature control, since the heating acts over the recycling line.

Figure 4.7 presents the process diagram of the pilot plant with indication of the most important elements and devices present. In Figure 4.8 a picture of the pilot plant location at the School of Engineering of the University of Santiago de Compostela (Spain) is given. It can be seen how the plant is built on a platform in order to make it relatively easy to transport to other location if needed.

4.4.2. Data acquisition, monitoring and control

The large number of on-line sensors present in the pilot plant motivated for the development and installation of an automatic monitoring, acquisition and control system. The software application (named *Acquirer*) is developed in Visual Basic for a PC platform that communicates with a PLC to whom all the sensors and actuators are connected.

Figure 4.9 presents the architecture of the information system in the plant. The on-line sensors are connected by analogical electric signals (typically 4-20 mA) to the PLC (Siemens S7/200 series) where the analogical signals are digitalised into a readable number. The PLC is connected to the PC by a serial cable converting protocols (RS485 to RS232) through which the digitalised information flows. The monitoring and acquisition software receives the values of the on-line measurements and they are displayed and stored in a database in the PC.

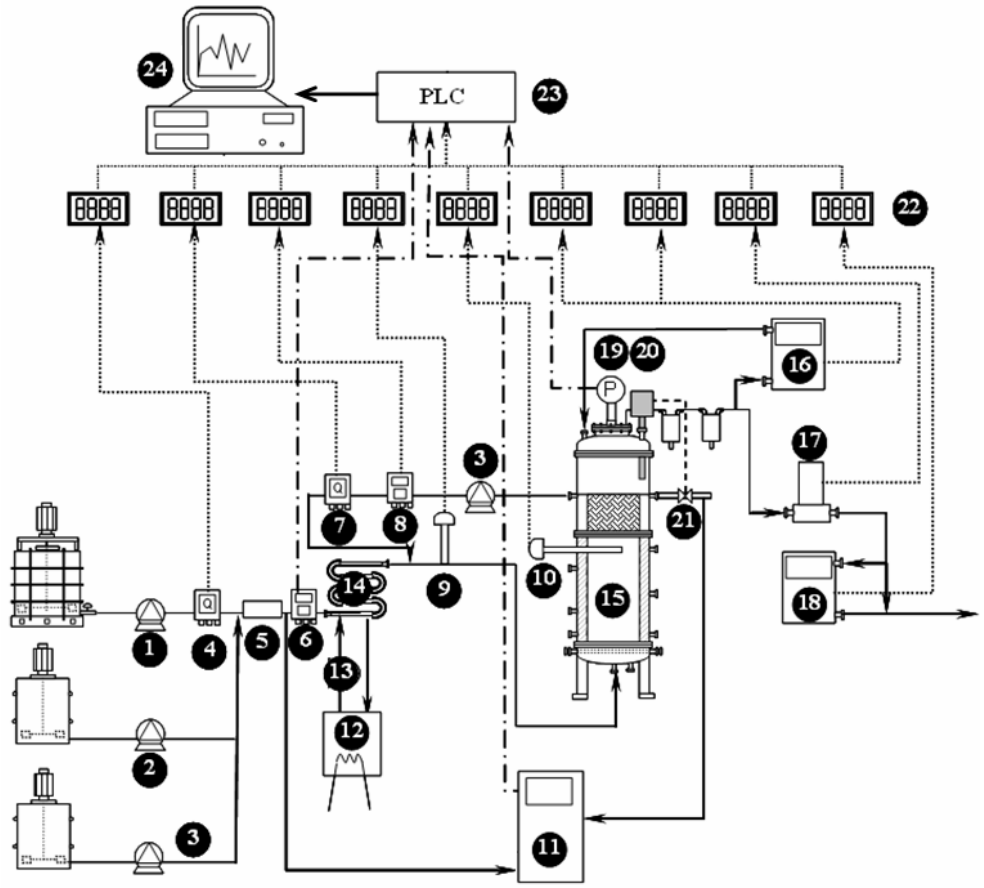

Figure 4.7. Process diagram of the pilot plant (adapted from Ruiz, 2005). Where (1) dilution pump, (2) nutrients pump, (3) concentrated feeding pump (wine), (4) dilution water flow meter, (5) static mixer, (6) feeding pH meter, (7) recycling flow meter, (8) recycling pH meter, (9) feeding temperature probe, (10) reactor temperature probe, (11) on-line TOC analyser, (12) water heater, (13) hot water pump, (14) heat exchanger, (15) hybrid UASB-AF reactor, (16) gas analyser (CH_4 y CO), (17) gas flow meter, (18) hydrogen analyser in the biogas, (19) pressure sensor, (20) level sensor, (21) effluent pneumatic valve, (22) displays panel, (23) PLC and (24) computer with the monitoring and control software. Water and gas lines (solid). Information lines (dotted).

Figure 4.8. Anaerobic pilot plant placed in the School of Engineering at the University of Santiago de Compostela. Hybrid UASB-AF reactor, pumps, piping, sensors, control panel, monitoring and data acquisition system.

The on-line monitoring and graphs windows interfaces are shown in Figure 4.10. The software is enough flexible to incorporate control algorithms and to connect with *Matlab* applications to perform advanced calculations for heuristic or optimal control purposes.

Advanced control algorithms can thus be implemented either in the code of the acquisition program or in *Matlab* depending on their nature and complexity. Once the control laws are applied for the current on-line measurements, the action values (e.g. a flow rate of a pump) are sent via (RS232/RS485) to the PLC and converted into analogical electrical signals to the corresponding actuation devices.

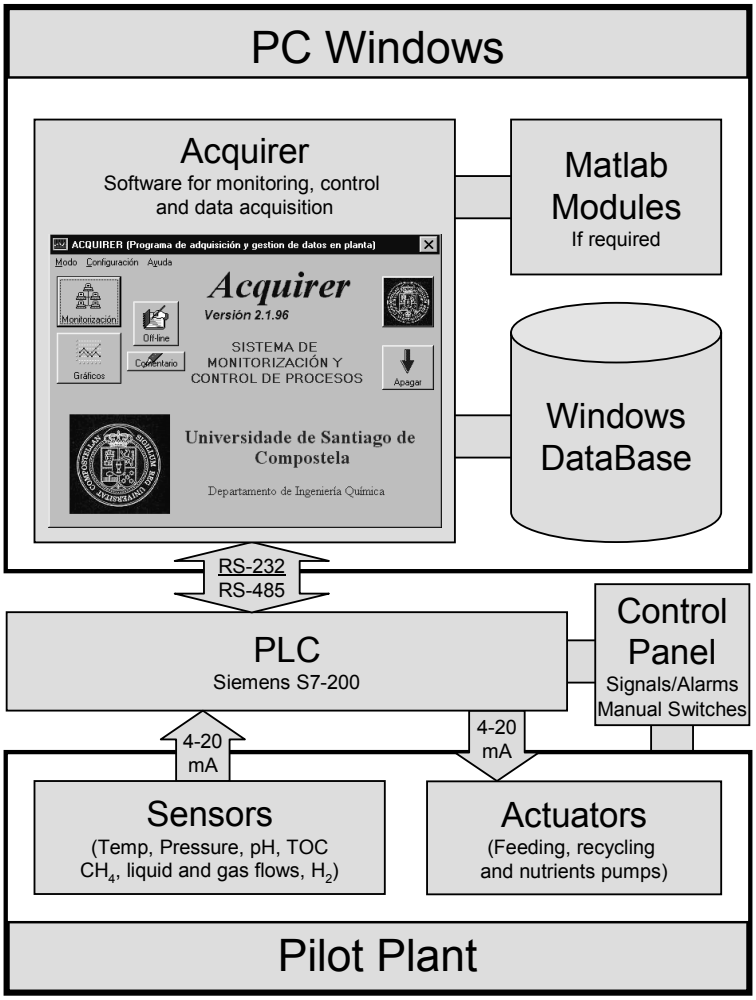

Figure 4.9. Architecture of the information system in the pilot plant.

Other simple routines, like disconnection protocols under alarm situations, are implemented directly in the PLC program, while some other manual switches and alarms are managed through the control panel.

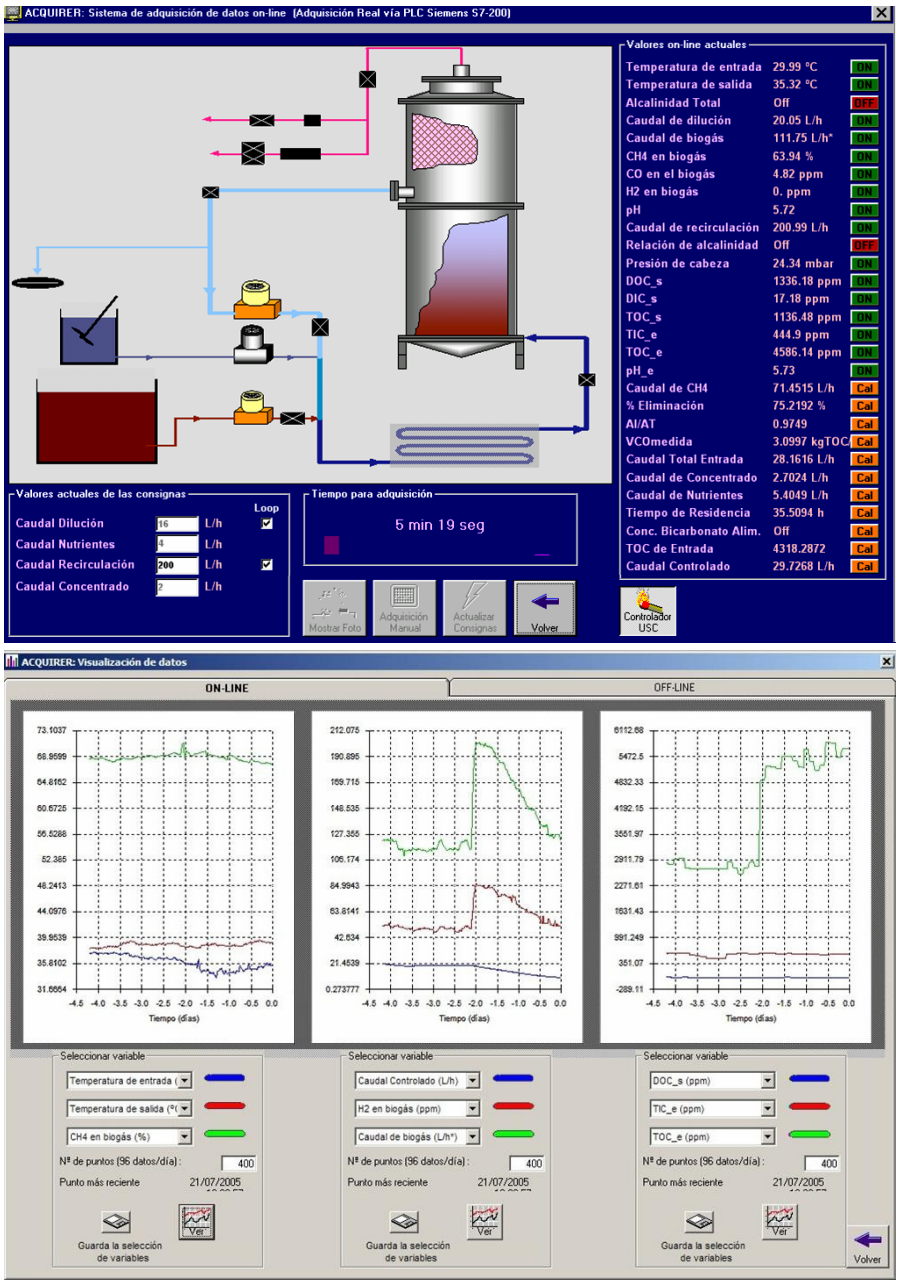

Figure 4.10. On-line monitoring and graphs interface windows of *Acquirer*.

4.4.3. Overload experiments with diluted wine

Several experiments were carried out in the fully instrumented hybrid UASB-AF digester described above (Ruiz, 2005). The pilot scale digester operated with tap water diluted wine as substrate and with nitrogen, phosphorous and bicarbonate addition as well. The reactor was operated at stable conditions for more than one month at an organic loading rate (OLR) of about 5 kgCOD/m^3·d.

Three consecutive increases of the OLR were applied in order to obtain three different constant treatment performances. Table 4.3 summarises the operational conditions imposed. The duration of each condition surrounded 5 days, time considered enough to achieve a pseudo-steady treatment performance (given that the HRT is between 0.6 and 1.5 d). During the last of the overloads (OLR of about 36 kgCOD/m^3·d) the process reached a complete inhibition of methanogenesis and an important accumulation of intermediate VFAs (up to 7 gVFA/L as acetic acid equivalents).

Table 4.3. Experimental conditions applied to the pilot scale UASB-AF reactor.

State	Period (d)	OLR (kgCOD/m3·d)	Feed Flow rate (L/h)	Influent TOC (mgC/L)
1: NO	≈ 0-4	≈ 5	22	≈ 3,000
2: HO	≈ 4-9	≈ 18	66	≈ 3,000
3: HO + OO	≈ 9-13	≈ 29	66	≈ 4,500
4: HO + OO	≈ 14-16	≈ 36	66	≈ 6,000

NO: Normal Operation; HO: Hydraulic Overload; OO: Organic Overload

4.5. Modification of the ADM1 for ethanol degradation

The IWA Anaerobic Digestion Model No.1 (ADM1) (Batstone *et al.*, 2002) provides a powerful tool for simulation and control of anaerobic digestion, which has been tested and applied recently (Batstone *et al.*, 2005). As indicated above, the ADM1, including only some processes selected by the workgroup, excluded ethanol degradation from the list of processes considered.

Considering that ethanol is a very reduced product, yielding stoichiometrically about 75% of methane and 25% of carbon dioxide, it has an immediate impact on the biogas composition respect to glucose. Intermediate conversions from ethanol give rise to different degradation products than from glucose, going through different pathways (Smith and McCarty, 1989). Ethanol is typically oxidised first to acetaldehyde and then to acetyl CoA. This activated compound is a central branch point of several pathways, leading to many fermentation products by CoA deactivation or other metabolic reactions.

The different ethanol degradation products motivated for the extension of the ADM1 carried out in this work as well as for enabling the application of the model to anaerobic digestion plants treating ethanolic wastewater from wine and breweries factories. The structural modification incorporated follows a fixed stoichiometry approach for ethanol acidogenesis for the sake of integration with the rest of the ADM1 structure.

The modification consisted of the addition of a new process for ethanol degradation by a new biomass group of ethanol degraders. A new process is added for the decay of the new biomass group as well. Figures 4.11a-b show the stoichiometry matrix of the ADM1 for the soluble and solid species with the modifications for ethanol treatment highlighted. The resulting model has 21 conversion processes and 26 species.

Two new state variables are added to the original ADM1, namely the concentrations of ethanol (S_{et}) and ethanol consumer biomass (X_{et}). The ethanol concentration is affected only by the ethanol uptake process (rS_{et}) while the ethanol consumer biomass concentration is affected by both the biomass growth by ethanol uptake through the biomass yield (Y_{et}) and by the decay process like the other biomass groups in ADM1.

A new kinetic expression is introduced for the ethanol uptake rate by the ethanol degrading biomass. It is assumed that this biomass group follows a Monod-type kinetic model for substrate limitation as proposed for the sugar consumers, namely the nitrogen-limitation-(I_{in_lim}) and general pH-inhibition-(I_{ph}) related terms, see Eq. 4-1.

$$rS_{et} = k_{m\_et} \cdot \frac{S_{et}}{K_{s\_et} + S_{et}} \cdot I_{in\_lim} \cdot I_{ph} \cdot X_{et} \qquad \text{Eq. 4-1}$$

	Component →	1	2	3	4	5	6	7	8	9	10	11	12	13
	Process ↓	Set	Ssu	Saa	Sfa	Sva	Sbu	Spro	Sac	Sh2	Sch4	Sic	Sin	Si
1	Disintegration												inN	fsi_xc
2	Hydrolysis of Carbohydr.		1											
3	Hydrolysis of Proteins			1										
4	Hydrolysis of Lipids		1-ffa_li		ffa_li									
5	Uptake of Ethanol	-1					(1-Yet)·fbu_et	(1-Yet)·fpro_et	(1-Yet)·fac_et	(1-Yet)·fh2_et		$v_{ic\ 5}$	-Yet·Nbac	
6	Uptake of Sugars		-1				(1-Ysu)·fbu_su	(1-Ysu)·fpro_su	(1-Ysu)·fac_su	(1-Ysu)·fh2_su		$v_{ic\ 6}$	-Ysu·Nbac	
7	Uptake of Amino Acids			-1		(1-Yaa)·fva_aa	(1-Yaa)·fbu_aa	(1-Yaa)·fpro_aa	(1-Yaa)·fac_aa	(1-Yaa)·fh2_aa		$v_{ic\ 7}$	Naa-Yaa·Nbac	
8	Uptake of LCFA				-1				(1-Yfa)·0.7	(1-Yfa)·0.3			-Yfa·Nbac	
9	Uptake of Valerate					-1		(1-Yc4)·0.54	(1-Yc4)·0.31	(1-Yc4)·0.15			-Yc4·Nbac	
10	Uptake of Butyrate						-1		(1-Yc4)·0.8	(1-Yc4)·0.2			-Yc4·Nbac	
11	Uptake of Propionate							-1	(1-Ypro)·0.57	(1-Ypro)·0.43		$v_{ic\ 11}$	-Ypro·Nbac	
12	Uptake of Acetate								-1		(1-Yac)	$v_{ic\ 12}$	-Yac·Nbac	
13	Uptake of Hydrogen									-1	(1-Yh2)	$v_{ic\ 13}$	-Yh2·Nbac	
14	Decay of Biomass Xet													
15	Decay of Biomass Xsu													
16	Decay of Biomass Xaa													
17	Decay of Biomass Xfa													
18	Decay of Biomass Xc4													
19	Decay of Biomass Xpro													
20	Decay of Biomass Xac													
21	Decay of Biomass Xh2													
		Ethanol (kg COD/m^3)	Monosaccharides (kg COD/m^3)	Amino Acids (kg COD/m^3)	Long Chain Fatty Acids (kg COD/m^3)	Total Valerate (kg COD/m^3)	Total Butyrate (kg COD/m^3)	Total Propionate (kg COD/m^3)	Total Acetate (kg COD/m^3)	Hydrogen (kg COD/m^3)	Methane (kg COD/m^3)	Inorganic Carbon (kmol-C/m^3)	Inorganic Nitrogen (kmol-N/m^3)	Soluble Inerts (kg COD/m^3)

Figure 4.11a. Stoichiometry matrix (soluble species part) of the modified ADM1 incorporating ethanol degradation process and its biomass decay.

	Component →	14	15	16	17	18	19	20	21	22	23	24	25	26
	Process ↓	Xc	Xch	Xpr	Xli	Xet	Xsu	Xaa	Xfa	Xc4	Xpro	Xac	Xh2	Xi
1	Disintegration	-1	fch_xc	fpr_xc	fli_xc									fxi_xc
2	Hydrolysis of Carbohydr.		-1											
3	Hydrolysis of Proteins			-1										
4	Hydrolysis of Lipids				-1									
5	Uptake of Ethanol					Yet								
6	Uptake of Sugars						Ysu							
7	Uptake of Amino Acids							Yaa						
8	Uptake of LCFA								Yfa					
9	Uptake of Valerate									Yc4				
10	Uptake of Butyrate									Yc4				
11	Uptake of Propionate										Ypro			
12	Uptake of Acetate											Yac		
13	Uptake of Hydrogen												Yh2	
14	Decay of Biomass Xet	1				-1								
15	Decay of Biomass Xsu	1					-1							
16	Decay of Biomass Xaa	1						-1						
17	Decay of Biomass Xfa	1							-1					
18	Decay of Biomass Xc4	1								-1				
19	Decay of Biomass Xpro	1									-1			
20	Decay of Biomass Xac	1										-1		
21	Decay of Biomass Xh2	1											-1	
		Composites (kg COD/m^3)	Carbohydrates (kg COD/m^3)	Proteins (kg COD/m^3)	Lipids (kg COD/m^3)	Ethanol Degraders (kg COD/m^3)	Sugar Degraders (kg COD/m^3)	Amino AcidDegraders (kg COD/m^3)	LCFA Degraders (kg COD/m^3)	Vale- & Butyrate Degraders (kg COD/m^3)	Propionate Degraders (kg COD/m^3)	AcetateDegraders (kg COD/m^3)	Hydrogen Degraders (kg COD/m^3)	Particulate Inerts (kg COD/m^3)

Figure 4.11b. Stoichiometry matrix (solid species part) of the modified ADM1 incorporating ethanol degradation process and its biomass decay.

No special attention is given in this work to the identification of the yield of biomass growth on ethanol independent from the kinetic parameters, since they are strongly correlated when the simulation starts from a steady state. A value for the biomass yield on ethanol is estimated using energetics. This fact is even more relevant when a high-solid-retention-time is considered as in this case. Even with good total biomass measurements available, no useful information for identification of these parameters could be obtained.

4.5.1. Ethanol degradation products

According to the results obtained from the highest overload experiment, four degradation products from ethanol are considered, namely butyrate, propionate, acetate and hydrogen. A scheme of the ethanol catabolic degradation proposed is shown in Figure 4.12.

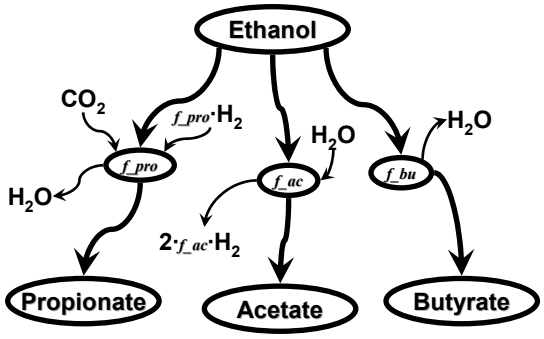

Figure 4.12. Scheme of the catabolic degradation of ethanol proposed.

Despite disadvantages have been reported (Kleerebezem and van Loosdrecht, 2004), the inorganic carbon is used to close the carbon balance, like in the original ADM1 model. The stoichiometric coefficients calculated are also assumed constant, but considering that the stoichiometry variability dependence on the environmental conditions is under study (Rodríguez *et al.*, 2004; Rodríguez *et al.*, 2005a).

4.5.2. Parameters of the ethanol degradation process

In order to define the stoichiometry of the ethanol degradation products, experimental data have been collected during a very high overload period with methanogenesis completely inhibited and pH value around 5. The apparent stoichiometry of the ethanol degradation process is obtained from measurements of the accumulation rates of butyrate, propionate and acetate during this overload period. The influent VFAs concentration remained negligible throughout the experimental period. Table 4.4 shows the estimated stoichiometric catabolic yields of ethanol into VFAs and hydrogen.

Table 4.4. Stoichiometric parameters estimated for the catabolic ethanol degradation.

Parameter	Value	Units
f_{bu_et}	0.167	kgCOD-Sbu/kgCOD-Set
f_{pro_et}	0.049	kgCOD-Spro/kgCOD-Set
f_{ac_et}	0.502	kgCOD-Sac/kgCOD-Set
f_{h2_et}	0.282	kgCOD-Sh2/kgCOD-Set

Considering that the observed activity is a combination of both the specific uptake rate and the concentration of specific biomass, there are important difficulties for the independent identification of both the biomass yield value of ethanol consumers and the specific maximum uptake rate of ethanol. Reasonable values for the biomass yield can be taken from the literature (Smith and McCarty, 1989; Seitz *et al.*, 1990) or they can be estimated from bioenergetic-related thermodynamic correlations, based on the amount of free energy dissipated by the metabolism of ethanol consumption (Heijnen, 1999). A value for the biomass yield is estimated from the energetics of the conversion pathway from ethanol to acetate. The conversion from ethanol to acetaldehyde and subsequently to acetyl CoA, with the final deactivation obtaining acetate and 1 ATP by substrate level phosphorylation, is considered. By comparing this energy yield of 1 ATP per mol of ethanol with the 4 ATP produced per mol of glucose (where glycolysis is involved) and converting to COD units, a biomass yield value for the growth on ethanol of half the value for growth on glucose is deduced. The value assumed for Y_{et} is therefore of 0.05 kgCODXet/kgCODSet.

With this value, the steady state of the model is computed for the operational conditions at which the sludge of the real plant developed and the steady state biomass composition, including the ethanol degraders, is estimated. With the steady biomass concentration and the total ethanol uptake rate observed during the overload period, a value for the maximum specific uptake rate of ethanol can be estimated. The changes in the biomass composition during the previous overloads are neglected and steady state is assumed.

The maximum ethanol uptake rate observed during the overload experiment is 23.8 kgCOD-EtOH/m^3·d and it is used to estimate a value for the specific maximum ethanol uptake rate (k_{m_et}). Considering that the pH value is around 5, after computing the steady state concentration of ethanol-consumer biomass for the given biomass yield and the pH inhibition term, a value for k_{m_et} of 3.6 kgCOD-S_{et}/kgCOD-X_{et}·d is taken. No parameter estimation is done for the Monod affinity constant (K_{s_et}) and a value of 0.1 kgCOD/m^3 is taken from the literature (Batstone *et al.*, 2004).

4.5.3. Simulation results

The overload experiments (see Table 4.3) are simulated after adjusting only the solid retention time of the reactor at a value of 100 days. The rest of parameters took the values recommended (Batstone *et al.*, 2002), except the low limit of pH inhibition for acetoclastic methanogens (pH_{LL_ac}), that was changed from 6 to 6.5. These pH inhibition parameters, as well as the structure of the inhibition function, appear as very sensitive, especially when adjusting the conditions at which the reactor breakdown occurs as in the case of the experiments simulated here (see Figure 4.13 and Figure 4.14).

The results of the simulations of the consecutive overloads with the modified model are presented in Figure 4.13 and Figure 4.14. The large difficulty in adjusting the system physicochemistry is blamed for the important differences encountered between measurements and simulated data. The high sensitivity of the breakdown point, at which the reactor methanogenesis inhibits completely, conditioned the adjustment of both the solid retention time and the low pH inhibition limit of acetoclastic methanogens and left almost no freedom for better fitting of the data.

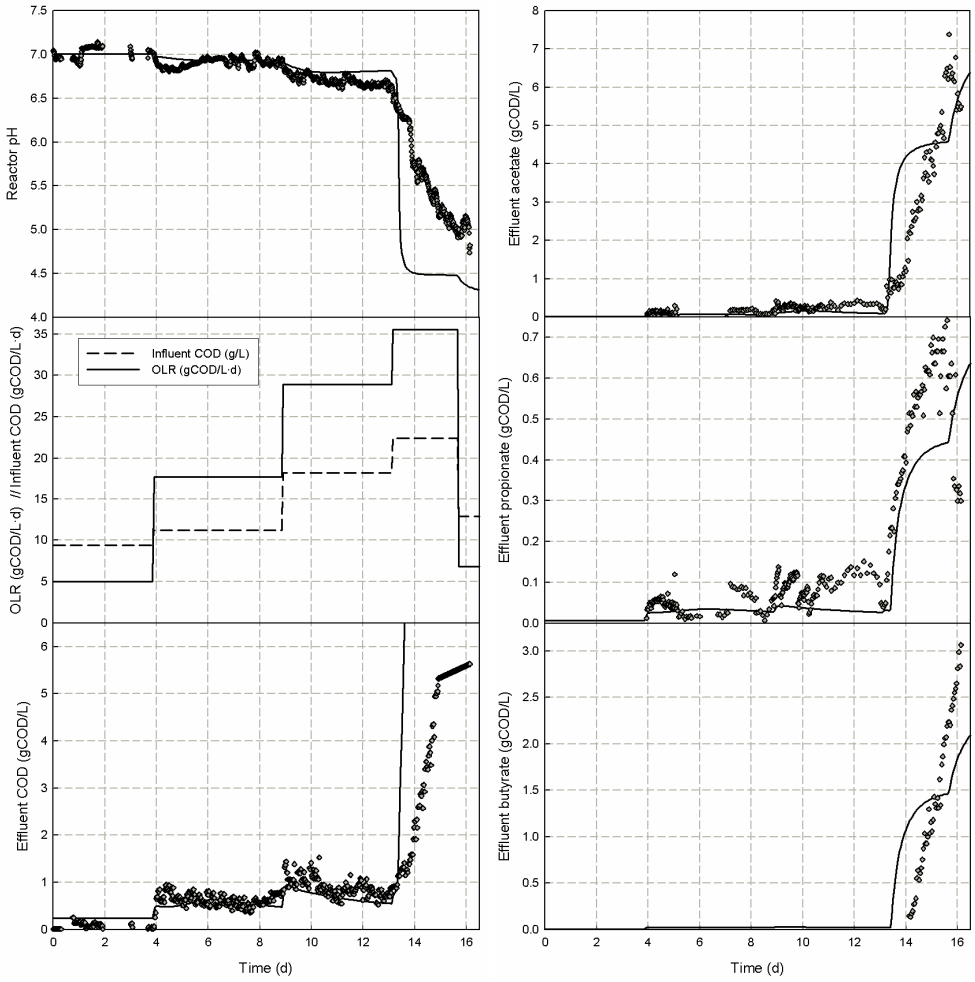

Figure 4.13. Simulation results with the ADM1 extended for ethanol treatment (pH, loading rate, influent and effluent COD and VFAs in the effluent).

Gas and methane production rates are acceptably well predicted as it usually happens since they are mainly dependent on the stoichiometry. The VFAs concentrations are well predicted as well specially during the final overload period from which the stoichiometric catabolic yields were estimated.

However, for the previous overload periods, the VFAs simulation results obtained are not so good. This suggests that, among the many factors normally affecting intermediate species, an important one could be an actual variable stoichiometry taking place, as opposite to the constant stoichiometry assumed in the model.

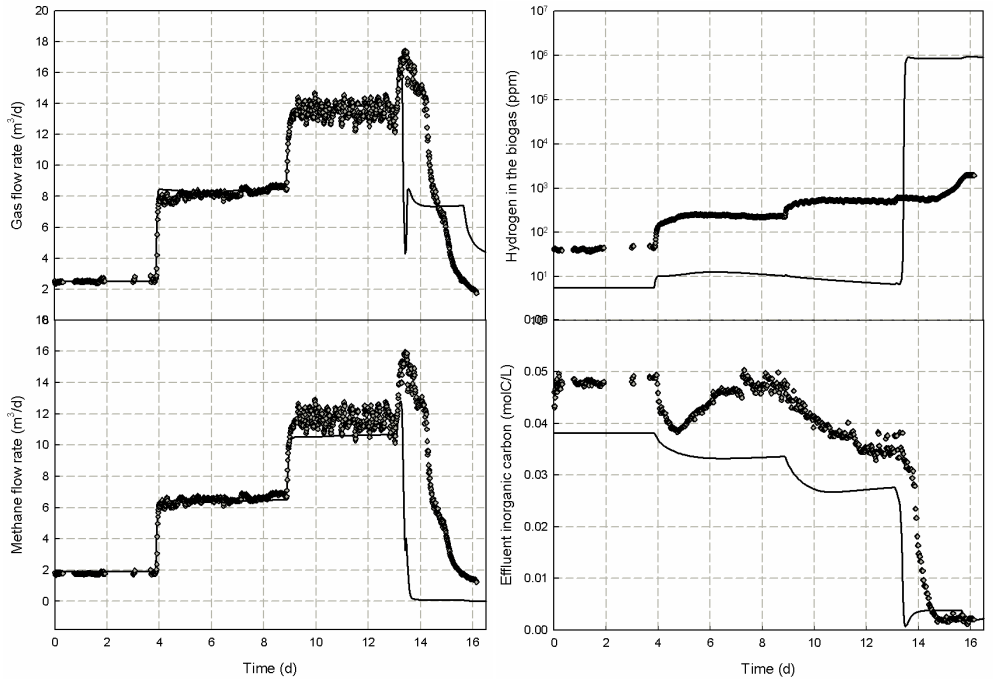

Figure 4.14. Simulation results with the ADM1 extended for ethanol treatment (gas and methane flow rates, hydrogen in the gas and inorganic carbon in the effluent).

The use of synthetic and well characterised wastewater made the predictions of the pH transitions also reasonably good, but discrepancy is found between the experimental and simulated concentration of inorganic carbon. Simulations of the effect of the k_La value assumed for the CO_2 transfer to gas phase showed that, below certain values, the k_La has an important impact on the inorganic carbon concentration and therefore on the physicochemical system. This, together with the differences between simulation and measurements during the final pH transition to very low values, suggest that gas transfer and some physicochemical parameters should be checked for this type of systems

The model predicts well the dynamics through the consecutive load increases up to the point where the system became overloaded and broke down. This confirms the suitability of ADM1 for dealing with dynamic situations where, even when the numeric values are not fully accurate, the dynamic behaviour is well reproduced. This makes a main interest of model for design and tuning of controllers.

Finally the system breakdown is observed to be very sensitive to the pH inhibition (that is a self amplifying process) as mentioned above. However small changes in the feed composition of alkalinity were checked and it was detected that small errors in the influent characterisation can cause the reactor breakdown to occur at a very different moment. This fact makes the selection of the proper pH inhibition parameters and the accuracy in the influent characterisation critic to get good results with ADM1 based models applied to systems pushed to the limit, like the case studied here.

Hydrogen levels in the biogas could not be predicted quantitatively at all. The sensitivity of this variable to any change in the model parameters and its stiffness in simulations is blamed for this problem. Hydrogen levels under stable operation have been identified as very characteristic from each system treating a certain wastewater. They must be assumed as a magnitude affected by many different phenomena and parameters as affinity constants, mass transfer issues, biomass composition, metabolic pathways, etc and they are recommended to be specifically determined for each particular system. This must be taken into account when hydrogen in the gas is the measured variable for control purposes (Ruiz, 2005), given its advantages as a fast response variable. Hydrogen based controllers should consider hydrogen levels under stable operation as characteristic from each different system and they should be experimentally determined for each specific case (Rodríguez *et al.*, 2005b).

4.6. Discussion and conclusions

The ADM1 model has been extensively applied in the last years since its publication. Some issues to be corrected as well as limitations to its applicability have been identified, considering that it was conceived as a general purpose model. The ADM1 structure with a fixed stoichiometry approach has some advantages as the relatively easy extension of model and modularity of the processes considered. The extensions reported, incorporated more processes to the model and are useful for particular applications. The discussion of which extensions should be incorporated remains open and depends on the definition of the model scope. The modular approach, enabling inclusion or exclusion of certain processes/variables, is an interesting alternative expanding the scope of the model for both general and specific systems in a simple way.

Some incorrect issues present in the current ADM1 can be easily corrected, as the case of the inorganic carbon used to close the carbon balances. To overcome this, the use of a mol-based instead of mixed COD-mol-based structure, is strongly recommended. This would both clarify the stoichiometry, avoiding unrealistic uptake of inorganic carbon for growth of certain microbial groups and also remove rounding errors of converting whole numbers, from reactions with well known stoichiometries, into COD units.

The detailed influent characterisation required remains as a big handicap for model applicability. Not only the level of detail about the influent composition is critical but also the accuracy of the characterisation measurements can strongly affect the model simulation results. This was studied above for overload experiments where the model output was proven to be very sensitive to small changes in the influent characteristics.

In addition to the limitation of the influent characterisation, the large number of parameters that need to be accurately estimated and/or identified is another weakness for the model applicability. Especially difficult is to tackle the identification of the maximum specific uptake rates separated from the biomass yield values. The experimental data available correspond in general to observed changes in concentration, which are affected by both the specific uptake rate and the particular biomass concentration. Moreover biomass concentrations are strongly dependent on the biomass yields and the decay constants used. Any experiment aiming at the determination of the specific kinetic

parameters must take this into account and explicitly indicate how far the observed kinetics are attributed to the kinetic process or rather to the concentration of active biomass present. It is frequent to find in the literature many different numeric values for kinetic parameters and a lack of discussion about their meaning in these terms above.

The extension of the ADM1 presented here introduced in the model both new kinetic and new stoichiometric parameters for the ethanol uptake process and its metabolism respectively. The estimation of the kinetic parameters was conducted linked to biomass yield, as explained above. The value of volumetric ethanol uptake observed is the result of the combination of the specific uptake rate and the concentration of ethanol-degrading biomass. The catabolic yields for acidogenesis products from the ethanol degradation were estimated during a highly overloaded period where methanogenesis was completely inhibited, situation at which the observed change in the intermediates can be attributed mainly to the ethanol conversion. This approach presents many limitations laying on the fact that these catabolic yields are expected to change with the environmental conditions as the pH or hydrogen concentration. This extended ADM1 is therefore recommended preferably for studies of transient situations as dynamic control purposes.

Apart from the quantitative outputs of the ADM1 based simulations, these general models normally provide interesting predictions of the dynamic transitions. These transitions are very dependent on time constants rather than on accurate parameter values and therefore less sensitive to certain rough parameter estimations. The ADM1 and extended versions are of great interest for dynamic applications as the design, tuning and validation of controllers. Advanced control techniques incorporating heuristics and statistics, together with classic control, can be tested and calibrated using the ADM1 as a powerful resource.

An issue that needs improvement is the applicability of the ADM1 to non methanogenic systems, i.e. pure fermentative processes. This is conditioned to the proper modelling of the acidogenic conversion and should be tackled from a variable stoichiometry perspective to stay as close as possible to the real behaviour.

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Chapter 5

VARIABLE STOICHIOMETRY IN ANAEROBIC DIGESTION MODELLING

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Chapter 5

VARIABLE STOICHIOMETRY IN ANAEROBIC DIGESTION MODELLING

Contents

Abstract

The incorporation of variable stoichiometry for acidogenesis of carbohydrates in the Anaerobic Digestion Model No.1 is studied in this chapter. The ADM1 is converted into a mol-based stoichiometry to enable easier implementation of these changes. The variable stoichiometry functions are derived from the results obtained by a previously developed mixed culture fermentation model. In methanogenic systems, the results show similar dynamic behaviour with the standard fixed-stoichiometry than with the variable-stoichiometry ADM1, due to the kinetic limiting methanogenesis. A variable stoichiometry with thermodynamic control is proposed for the acidogenic and acetogenic steps.

5.0. Summary

Most existing anaerobic digestion models, including the IWA Anaerobic Digestion Model No.1 (ADM1), consider a fixed-stoichiometry for their conversion processes. In this chapter the effect of incorporating variable stoichiometry in the carbohydrate fermentation process of the ADM1 is investigated. The ADM1 model is firstly transformed into a molar-based model to avoid some errors derived from the mixed COD-mol-based standard model and to facilitate the implementation of the variable stoichiometry. Consequently, the values of the butyrate and acetate catabolic yields of carbohydrate fermentation are calculated as a function of the hydrogen concentration and the reactor pH, according to the predictions of the mixed culture fermentation model presented in Chapter 2, built upon thermodynamic and energy-related issues.

The simulation results obtained showed no significantly different responses, in terms of effluent quality and system robustness, between the standard and the variable stoichiometry ADM1 under overload conditions, for both single- and two-step anaerobic digestion configurations. This behaviour is explained by the non limiting acetogenic activity that compensated for the changes in the acidogenic products, typical behaviour for serial processes close to equilibrium. Based on the results obtained, thermodynamic rather than kinetic control for these conversions is suggested. Depending on the objectives to be met, lumping of carbohydrate fermenters and oxidative acetogens into a single biomass group with a variable stoichiometry is proposed for further consideration.

5.1. Introduction

Most of the existing models of anaerobic digestion, including ADM1 (Batstone *et al.*, 2002), consider fixed-stoichiometry for their conversion processes (Vavilin *et al.*, 1995; Kalyuzhnyi, 1997; von Munch *et al.*, 1999). Many of these models use kinetic-based approaches to fit the experimental behaviour, incorporating inhibition terms when needed and leading sometimes to a large number of parameters with questionable mechanistic interpretation. Some of this type of models inherited model structures from the ASM family models (Henze *et al.*, 1987; Gujer *et al.*, 1999), originally developed for activated sludge systems whose processes are more clearly kinetically controlled, provide high energy yields and proceed far from thermodynamic equilibrium.

Anaerobic processes however, as opposed to aerobic processes, provide low energy yields and their conversions can proceed very close to thermodynamic equilibrium (Kleerebezem and Stams, 2000), this suggests that anaerobic processes may be controlled thermodynamically rather than kinetically.

In anaerobic digestion the step of carbohydrates fermentation (acidogenesis), by fermenting microbial populations, leads to a variety of products that are subsequently methanised by other microbial populations. It is known moreover that the type of fermentation products obtained is influenced by the operational conditions (Zoetemeyer *et al.*, 1982a; Zoetemeyer *et al.*, 1982b; Horiuchi *et al.*, 2002), showing a clear variable stoichiometry. Recently the carbohydrate fermentation process implemented in the ADM1 (Batstone *et al.*, 2002) has been identified as an issue demanding further development (Batstone *et al.*, 2005).

The increasing interest in carbohydrate fermentation technologies for production of chemicals (Reis *et al.*, 2003; Dürre, 1998) and energy carriers (Claassen *et al.*, 1999; Benemann, 1996) demands for developing better models of these processes. The investigation conducted here contributes to the development of mechanistic models for prediction of the fermentations products from carbohydrates in mixed culture systems and to their integration into existing models as the ADM1.

The prediction of the product spectrum obtained during fermentation of carbohydrates by mixed cultures has been studied in the past decades and some models have been proposed (Mosey, 1983; Costello *et al.*, 1991). Some of these models applied a fixed stoichiometry to the fermentation of carbohydrates and others intended to predict the effect of hydrogen on the product scheme from a thermodynamic point of view. These authors demonstrated that the product spectrum can be very diverse depending on the environmental conditions.

The steady state mixed culture fermentation (MCF) model developed in Chapter 2 (Rodríguez *et al.*, 2004; Rodríguez *et al.*, 2006) focuses on the prediction of the product spectrum as a function of the environmental conditions of cultivation. The model intends to predict the product fluxes based on the optimum performance of the mixed culture in terms of growth, limited by energy as ATP, and considering the bioenergetics of the microbial conversions and growth.

In anaerobic digestion, the carbohydrate fermentation products are the substrates for the acetogenic and methanogenic biomass. The composition of those products affects therefore the composition of these biomass groups. The sludge composition and activity should be different depending on the acidogenic product spectrum obtained. Different sludge characteristics should lead to different steady state and dynamical performance under disturbances.

This work focuses on the study of the implications of considering a variable product stoichiometry for glucose fermentation, in the ADM1 predictions. Several simulations are conducted to analyse the effect of changing dynamically the stoichiometry of product formation in the carbohydrate fermentation process, using the stoichiometry changes predicted by the recently developed MCF model (see Chapter 2). By incorporation of this variable stoichiometry, the possibility of redefining certain steps of the anaerobic digestion towards a thermodynamic-controlled variable-stoichiometry model may be studied.

5.2. Adaptation to a molar-based ADM1

In order to overcome some inaccuracies identified in the ADM1 (Kleerebezem and van Loosdrecht, 2004), regarding the consequences of using both COD and molar units together within the same model, all the COD state variables of the ADM1 are redefined into molar units and the corresponding kinetic and stoichiometric parameter values properly adapted. Elemental and charge balances are closed for all the processes considered as well.

Table 5.1. Elemental composition of the species of the mol-based ADM1.

Species	Abbr.	C	H	O	N	Charge	γ (e-/Cmol)	COD (gCOD/mol)
Glucose	Ssu	6	12	6	0	0	4	192
Aminoacids (lumped)	Saa	1	1.9	0.6	0.2253	0	4.0240	32.1918
LCFA	Sfa	1	1.9	0.1	0	0	5.7	45.6
Valeric acid	Sva	5	10	2	0	0	5.2	208
Butyric acid	Sbu	4	8	2	0	0	5	160
Propionic acid	Spro	3	6	2	0	0	4.6667	112
Acetatic acid	Sac	2	4	2	0	0	4	64
Hydrogen	Sh2	0	2	0	0	0	0	16
Methane	Sch4	1	4	0	0	0	8	64
Carbon dioxide	Sic	1	0	2	0	0	0	0
Amonium	Sin	0	3	0	1	0	0	0
Soluble inerts	Si	1	1.9460	0.6754	0.1429	0	4.1667	33.3333
Particulate substrate	Xc	1	1.5	0.5324	0.0895	0	4.1667	33.3333
Solid carbohydrates	Xch	1	2	1	0	0	4	32
Solid protein	Xpr	1	1.9	0.6	0.2253	0	4.0240	32.1918
Solid lipids	Xli	1	1.9	0.1	0	0	5.7	45.6
Glucose fermenters	Xsu	1	1.4	0.4	0.2	0	4	32
Aminoacid oxidizers	Xaa	1	1.4	0.4	0.2	0	4	32
LCFA oxidizers	Xfa	1	1.4	0.4	0.2	0	4	32
Butyrate oxidizers	Xc4	1	1.4	0.4	0.2	0	4	32
Propionate oxidizers	Xpro	1	1.4	0.4	0.2	0	4	32
Acetoclastic methanogens	Xac	1	1.4	0.4	0.2	0	4	32
Hydrogenotrophic methanogens	Xh2	1	1.4	0.4	0.2	0	4	32
Solid inerts	Xi	1	1.4	0.4024	0.1429	0	4.1667	33.3333

Table 5.1 shows the elemental composition considered of the mol-based ADM1 species. Protein and aminoacids, lipids, LCFA and inerts composition may vary depending on the wastewater considered. The value γ is the number of electrons per carbon mol available to be donated to electron acceptors (Heijnen, 1999), each electron donated equals 8 gCOD.

After adaptation of the parameters to mol units the stoichiometries of the processes of the mol-based ADM1 are calculated (see Table 5.2). When converting to mol-based units, major differences arose in the hydrogen yields from valerate (+15.58%) and butyrate degradation (+8.11%) respect to the COD-based ADM1 values (Batstone *et al.*, 2002). The unrealistic use of inorganic carbon to close the carbon balances in the COD-based ADM1 is blamed for these differences (Kleerebezem and van Loosdrecht, 2004).

Other minor numeric differences are found and attributed to rounding errors when converting mol-based (with whole numeric stoichiometry coefficients) into COD-based (with fractional numeric coefficients) reaction stoichiometries in the standard ADM1. Particularly, the hydrogen yield from propionate (+1.29%) and the acetate yield from propionate (+0.25%) are different from the standard COD-based ADM1.

Table 5.2. Mol-based stoichiometry of the ADM1 bioconversions.

Process / Species	Disint.	Carb. Hydrol.	Proteins Hydrol.	Lipids Hydrol.	Gluc. Ferm.	Amin. Oxidat.	LCFA Oxidat.	Valer. Oxidat.	Butyr. Oxidat.	Prop. Oxidat.	Acetocl. Methan.	Hydrog. Methan.	Biomass Decays
Ssu	0	0.1667	0	0.0119	**-1**	0	0	0	0	0	0	0	0
Saa	0	0	1	0	**0**	-1	0	0	0	0	0	0	0
Sfa	0	0	0	0.95	**0**	0	-1	0	0	0	0	0	0
Sva	0	0	0	0	**0**	0.0327	0	-1	0	0	0	0	0
Sbu	0	0	0	0	**0.1404**	0.0481	0	0	-1	0	0	0	0
Spro	0	0	0	0	**0.4166**	0.0132	0	0.922	0	-1	0	0	0
Sac	0	0	0	0	**1.107**	0.1850	0.4561	0.922	1.85	0.9533	-1	0	0
Sh2	0	0	0	0	**2.052**	0.1120	0.8546	2.078	2	2.9067	0	-1	0.0257
Sch4	0	0	0	0	**0**	0	0	0	0	0	0.95	0.235	0
Sic	0.0653	0	0	-0.0213	**1.3747**	0.1538	0.0023	0	0	0.9533	0.95	-0.265	0.0523
Sin	0.0000	0	0	0	**-0.12**	0.2092	-0.0171	-0.078	-0.06	-0.028	-0.02	-0.006	0.1152
Si	0.1	0	0	0	**0**	0	0	0	0	0	0	0	0
Xc	-1	0	0	0	**0**	0	0	0	0	0	0	0	0.9477
Xch	0.2083	-1	0	0	**0**	0	0	0	0	0	0	0	0
Xpr	0.2071	0	-1	0	**0**	0	0	0	0	0	0	0	0
Xli	0.2193	0	0	-1	**0**	0	0	0	0	0	0	0	0
Xsu	0	0	0	0	**0.6**	0	0	0	0	0	0	0	-1
Xaa	0	0	0	0	**0**	0.0805	0	0	0	0	0	0	(-1)
Xfa	0	0	0	0	**0**	0	0.0855	0	0	0	0	0	(-1)
Xc4	0	0	0	0	**0**	0	0	0.39	0.3	0	0	0	(-1)
Xpro	0	0	0	0	**0**	0	0	0	0	0.14	0	0	(-1)
Xac	0	0	0	0	**0**	0	0	0	0	0	0.1	0	(-1)
Xh2	0	0	0	0	**0**	0	0	0	0	0	0	0.03	(-1)
Xi	0.2	0	0	0	**0**	0	0	0	0	0	0	0	0

The use of mol-based units enables to set the well defined stoichiometry of some reactions without rounding errors and unrealistic values. Mol units facilitate as well thermodynamic and energetic calculations and thus the implementation of the variable stoichiometry approach.

5.3. Variable stoichiometry for carbohydrate fermentation

In order to analyse the implications of the inclusion of a variable stoichiometry in the carbohydrate fermentation process of the ADM1, a function changing dynamically the stoichiometry matrix is implemented. The results from the mixed culture fermentation (MCF) model developed in Chapter 2 (Rodríguez *et al.*, 2004; Rodríguez *et al.*, 2006) are used to define the function. The model predicts the dependence of the fermentation products as a function of the pH and the hydrogen concentration in the reactor.

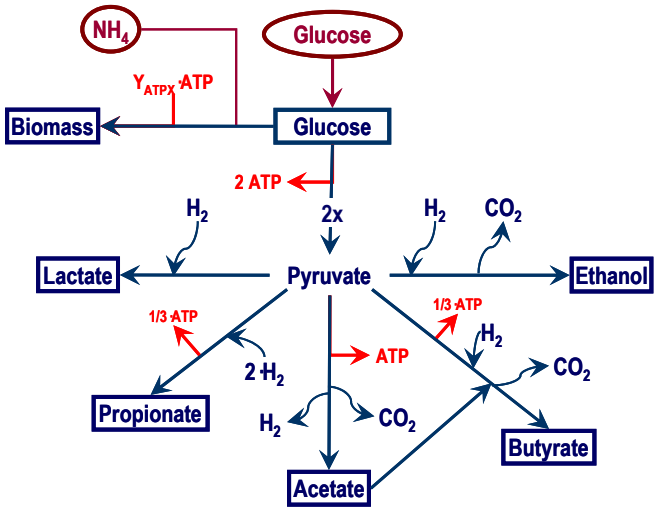

Figure 5.1. Simplified metabolic network of the carbohydrate fermenting biomass

The MCF model developed considers a virtual microorganism with the most common carbohydrate fermentation pathways and their bioenergetics, implemented in a metabolic network (see Figure 5.1). The maximized growth of that virtual fermentative microorganism defines the product spectrum. The model assumes thermodynamic control rather than kinetic, supported on the fact that anaerobic processes run very close to equilibrium (Kleerebezem and Stams, 2000). The MCF model used needs further development and experimental validation but provides a qualitative behaviour that can be applied to analyse the effect of dynamic changes in the stoichiometry of the ADM1.

The main change predicted by the MCF model, was a shift from acetate to butyrate as main fermentation product at decreasing pH (7 to 5.5) and/or increasing hydrogen concentration in the liquid. Note that ADM1 considers the supersaturation in the liquid phase, caused by mass transfer limitation of hydrogen to the gas phase. The hydrogen concentration in the liquid depends both on the hydrogen partial pressure and on the liquid-gas transfer rate.

Among the predictions of the MCF model the stoichiometry change between butyrate and acetate is the most important. Only this stoichiometry variation is considered here to conduct the study as simple as possible. The stoichiometry change functions derived are the result of integrating the MCF results within the standard ADM1 model structure. Figure 5.2 shows this stoichiometry variability as a function of the dissolved hydrogen concentration and the reactor pH.

The integration of the MCF model predictions in ADM1 is achieved by the derivation of continuous functions describing the stoichiometry variation. They start at the standard ADM1 values (Batstone *et al.*, 2002) that apply for low hydrogen concentrations and non acid pH. Just the ratio acetate/butyrate predicted by the MCF model predictions is used, keeping constant at the standard ADM1 value the total amount of carbon to butyrate plus acetate and closing the COD balance with the hydrogen yield. The standard catabolic yield of propionate was kept constant.

The functions of stoichiometric change of the catabolic yields (see Figure 5.2) show how either at low pH and/or high hydrogen concentration, the catabolism shifts to butyrate as main product. A mechanistic explanation of this prediction of the MCF model is related to energetic issues. Lower pH values required higher energy costs for the cells to transport acid molecules outwards the membrane and on the other hand, the maximum concentration of a product is limited by the thermodynamic feasibility of its production where hydrogen plays a key role, especially in the acetate production. The combination of both effects justifies for the shift to butyrate production since one molecule of acetate is incorporated per butyrate produced and then does not have to be transported outwards and no net hydrogen is produced (see Figure 5.1).

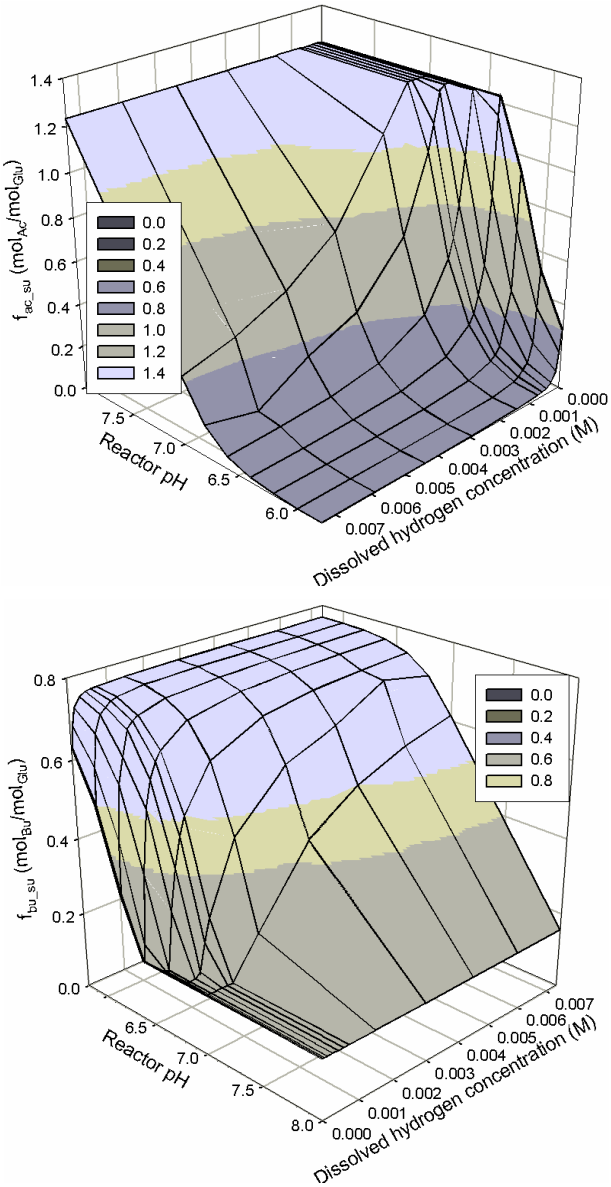

Figure 5.2. ADM1 catabolic stoichiometry coefficients of acetate and butyrate as a function of the dissolved hydrogen concentration and the reactor pH during mixed culture glucose fermentation. (Note the different axis layout)

The variable stoichiometry is implemented in ADM1 only for the carbohydrate fermentation process. The stoichiometry change is calculated during the simulation at any time step by evaluating the surfaces from Figure 5.2 by linear interpolation of the two variables function, namely the given actual pH value and the dissolved hydrogen concentration in the liquid.

5.4. Variable stoichiometry in methanogenic reactors

Simulations of a methanogenic anaerobic chemostat reactor are conducted to analyse the effect of a variable stoichiometry in the carbohydrate fermentation. The initial state comes from a steady state performance with an influent glucose concentration of 10 g/L, 75 mM of bicarbonate to maintain neutral pH preventing inhibition effects and nitrogen source guaranteeing for no nitrogen limitation at any time of the simulations. A high and constant HRT of 100 h maintains methanogens within the reactor.

The steady state biomass composition obtained (see Table 5.3) shows no difference between fixed and variable stoichiometry models because at neutral pH, hydrogen concentration remains low in a methanogenic system. The stoichiometric catabolic yields remain thus at the standard ADM1 values where production of acetate over butyrate is favoured.

Table 5.3. Steady state biomass composition of the chemostat simulation.
(HRT = 100 h; Ssu_in = 10 g/L; pH = 7)

Units	Xc	Xch	Xpr	Xli	Xsu	Xaa	Xfa	Xc4	Xpro	Xac	Xh2	Xi
molCx/L	0.00123	0.00001	0.00001	0.00001	**0.03068**	0	0	**0.00205**	**0.00278**	**0.00643**	**0.00529**	**0.00051**
gCODx/L	0.03920	0.00040	0.00038	0.00042	**0.98188**	0	0	**0.06546**	**0.08880**	**0.20564**	**0.16914**	**0.01633**

To analyse the effect of the variable stoichiometry, it is necessary to disturb the system causing a situation where the stoichiometry varies according to the functions implemented. A 2-days-long influent glucose concentration overload of 300 g/L is applied to compare results between the two models, causing a pH decrease and hydrogen increase that led to a strong stoichiometry change.

Figure 5.3 presents the responses obtained by the fixed and the variable stoichiometry models. In both cases the hydrogen concentration increases initially when its production rate exceeds the capacity of hydrogenotrophic methanogens. After about 20 hours the hydrogen partial pressure decreases because of the enhanced hydrogen uptake capacity due to hydrogenotrophs growth under non inhibitory pH levels. Due to the high HRT used, a significant drop in the pH as a result of VFA accumulation occurs after approximately 20 h, resulting in strong inhibition of the hydrogenotrophic methanogens and accumulation of hydrogen in the gas phase during the overload.

The major difference between fixed and variable stoichiometry simulations appears in the effluent composition during the overload. Despite the production of more butyrate instead of acetate leads to less number of acid molecules, the pH breakdown is not significantly delayed with respect to the fixed stoichiometry case. Effluent quality, in terms of COD and recovering time after the overload, is not significantly affected. This is clearly attributed to the rate limiting conversion of acetate to methane and non rate limiting conversion of butyrate to acetate.

This fast dynamics suggests that the acidogenic and acetogenic conversions remain close to thermodynamic equilibrium and are thermodynamically rather than kinetically controlled. When both acidogenic and oxidative acetogens are present, the effect of changing acidogenic stoichiometry is immediately compensated by the activity of acetogens, according to ADM1 structure and parameters, and the effluent characteristics remain almost unchanged.

The variable stoichiometry thermodynamic controlled model behaves therefore analogous to the fixed stoichiometry kinetic controlled model under steady state conditions in methanogenic systems. Differences appear however when lower pH or higher hydrogen concentration occur but only in the effluent composition during the transient periods.

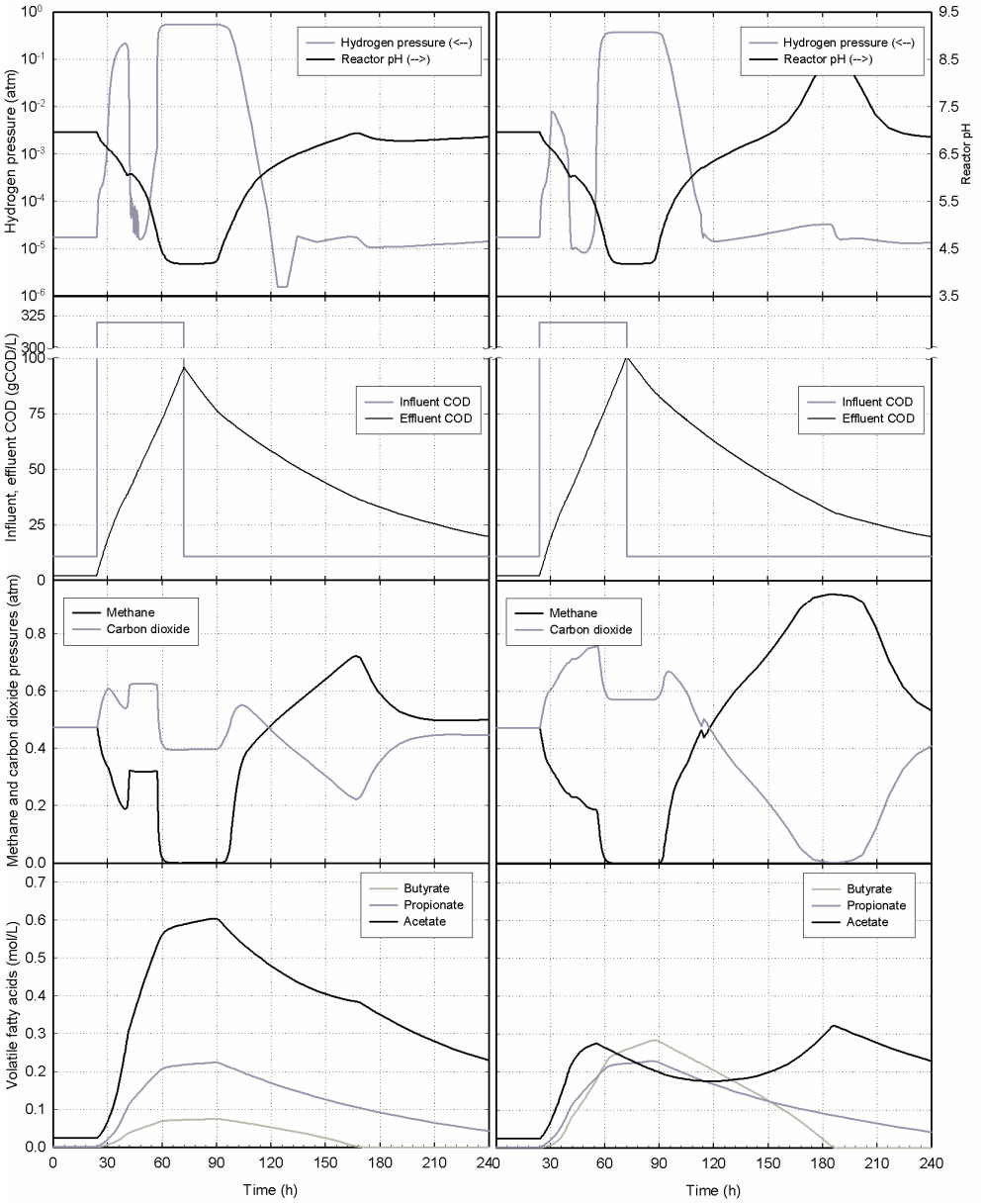

Figure 5.3. Dynamic response of a methanogenic chemostat reactor (HRT of 100 h) to an overload, using fixed stoichiometry (left) and variable stoichiometry (right) ADM1.

5.5. Variable stoichiometry in two step anaerobic digestion

In addition to the methanogenic, a two step anaerobic digestion system was simulated to compare the steady states of both the acidogenic and methanogenic reactors when using the fixed and the variable stoichiometry models. The acidogenic reactor simulated is a chemostat with a HRT of 9 h and the methanogenic reactor has a HRT of 3.6 h and SRT of 10 days. The comparison between both fixed and variable stoichiometry steady states is presented in Figure 5.4 for the acidogenic and methanogenic reactors.

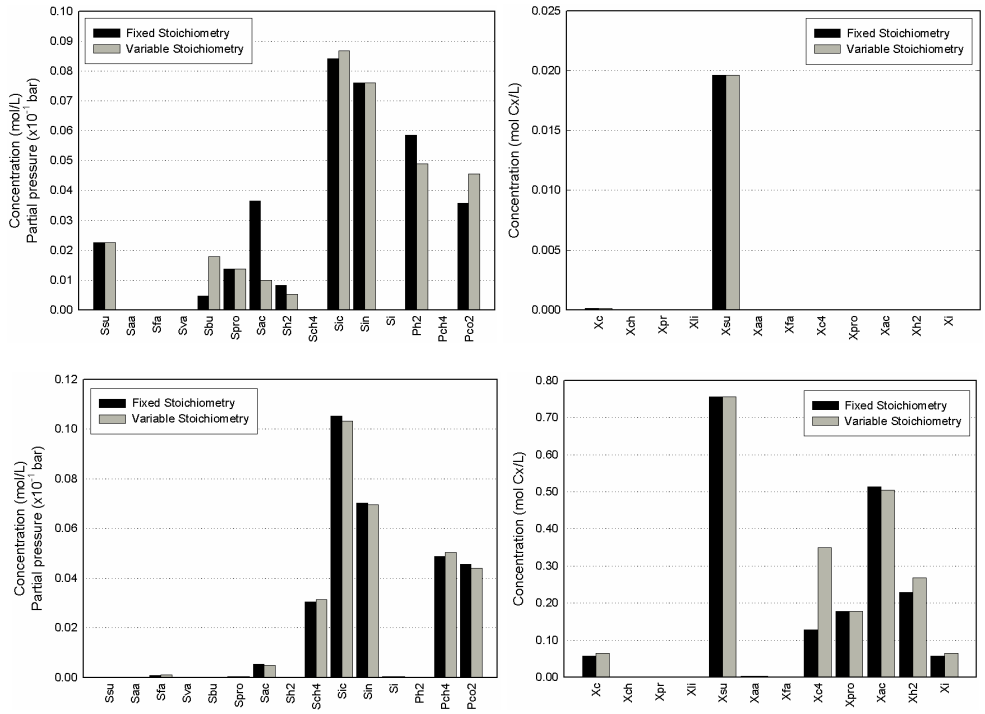

Figure 5.4. Steady states of the acidogenic (top) and methanogenic (bottom) reactors. Comparison between fixed and variable stoichiometry models.

The absence of hydrogenotrophs methanogens in the acidogenic reactor causes the stoichiometry change due to the high hydrogen pressure achieved. With the short SRT of 9 hours only carbohydrate fermenters stay in the acidogenic reactor and the biomass

composition does not vary between the two models. The effluent composition (influent to the methanogenic) presents however a much higher ratio butyrate/acetate when the variable stoichiometry is used, due to the very high hydrogen pressures occurring in a fermenter.

Despite the major differences in its influent composition, the methanogenic reactor shows only minor changes in the effluent composition. The biomass composition, not directly measurable in practice, presents major difference in the concentration of oxidising bacteria from butyrate, due to the higher butyrate/acetate ratio in the influent.

This similar effluent composition obtained using both fixed or variable stoichiometry is again due to the rate limiting methanogenesis and non limiting acetogenesis, showing that acido- and acetogenesis are not kinetically controlled.

5.6. Discussion and conclusions

The simulations, comparing the fixed stoichiometry standard ADM1 with a modified model incorporating variable stoichiometry for carbohydrate fermentation, led to very similar steady state and dynamic results in terms of COD elimination. This is caused by the rate limiting methanogenesis and suggests that acidogenic and acetogenic conversions are not kinetically controlled. In methanogenic systems the standard model behaved similar to a variable stoichiometry conversion, controlled by thermodynamics instead of by kinetics (Rodríguez *et al.*, 2006).

Implementation of a variable stoichiometry biomass in the acidogenic reactor would improve the model applicability to these systems. A variable stoichiometry system, with variable acidogenesis products in the reactor effluent and variable biomass yield, as expected to occur experimentally (Smith and McCarty, 1989), would provide a mechanistic interpretation of the actual variable product formation in non methanogenic fermentations.

With this approach the kinetic term used for hydrogen inhibition of oxidative acetogens used in the ADM1 could be implicitly incorporated as a more realistic change in the acidogenesis products composition when high hydrogen levels occur. This avoids the computational problems associated with the use of thermodynamic shift functions. Other phenomena as the kinetic inhibitory pH effect could be treated as a transport-related energetic maintenance effect, independent from growth.

The thermodynamically based approach for prediction of the acidogenic products could be also useful when applying the model to other substrates than glucose, like ethanol or other carbohydrates. The prediction of the product spectrum should be based on the same thermodynamic and transport-related energetic issues. Thus the applicability of the model to fermentative systems with a wider spectrum of substrates can be extended more easily. Implementation of the variable stoichiometry model used here is not only of relevance for methanogenic systems and is being developed for general mixed culture fermentations of carbohydrates.

Some recent works using statistic tools indicate that an anaerobic digestion system can present a smaller variability (Bernard *et al.*, 2005). Lumping or redefinition of some conversions could be suggested specifically when the model is not focused on biomass prediction. Depending on the model objectives and when focused mainly on prediction and identification of liquid and gas phase behaviour, a thermodynamic controlled lumped biomass approach with variable stoichiometry could be considered.

It is important to note that the dynamic results obtained assume that the stoichiometry changes occur instantaneously because the stoichiometry functions do not incorporate any time related issue. This is not assumed as realistic but only used at this point for testing what the effect of such a dynamic stoichiometry change would be, in the most extreme situation of instantaneous change of the stoichiometry. Slow stoichiometry changes may occur in reality but similar results are expected maybe with minor time delays or small variations.

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Chapter 6

ASSESSING THE MODEL COMPLEXITY FOR ANAEROBIC BIOPROCESSES

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Chapter 6

ASSESSING THE MODEL COMPLEXITY FOR ANAEROBIC BIOPROCESSES

Contents

Abstract

A methodology using principal component analysis (PCA) to assess the number of relevant reactions from experimental data, applicable both for structure characterisation and simplification of models, is presented. The methodology is applied to experimental data from two types of anaerobic reactors and wastewaters. Four reactions appear to represent almost completely the variability of all these experiments. The PCA-based methodology is applied also to data provided by simulations with the complex ADM1 for model reduction purposes and the results show that also four reactions describe the variability of the simulation data. This suggests that a number of four lumped conversions is sufficient for description of these types of anaerobic digestion systems, provided that conversions proceed according to a fixed stoichiometry.

6.0. Summary

In this chapter a methodology, based on principal component analysis (PCA), to assess the number of relevant reactions is presented. It is applied to experimental and simulated data from anaerobic digestion processes.

General expressions of mass balances are mathematically transformed and separated in two terms, from which one is independent from kinetics and can be computed from experimental measurements. The transformed and normalised data are integrated and filtered to remove excess noise and the PCA-based method is applied. The percentage of variability versus the number of reactions considered is obtained. Each reaction corresponds to a principal component obtained and retains part of the system variability.

Using this PCA-based methodology, an *a priori* model structure characterisation technique is proposed. Once the optimum number of reactions to be considered in the model structure is assessed, the modeller's expertise and the information extracted from the principal components allow candidate model structures to be set up.

A technique for simplification of complex models is also presented using the PCA-based methodology. The PCA can be applied to simulation data generated with a complex model, under the conditions the simplified model is to be utilised. The PCA analysis provides the number of relevant reactions occurring and allows the modeller to propose an alternative simplified model structure, using prior knowledge and the information from the principal components. In this case the existing structure of the complex model provides also valuable guidelines to propose the simplified structure of the reduced model.

The PCA-based methodology is applied to experimental data obtained from the operation of a pilot scale UASB-AF anaerobic reactor treating diluted wine and from a CSTR reactor treating industrial wastewater from a distillery plant. From the assessment of the system dimension using PCA it is concluded that with only 4 reactions, the variability of these systems is almost fully described. The PCA-based methodology is applied also to ADM1 simulation data of these systems and also 4 reactions appear as enough to describe the variability shown by the dynamic simulation data. 4 processes are therefore recommended for anaerobic digestion models within the range of experimental conditions studied.

6.1. Introduction

Mathematical models are widely used in the design, optimisation and operation of bioprocesses, including biological wastewater treatment systems. A mechanistic model of a biological system is expected to describe reality as accurate as possible and maintaining the biological interpretation of its parameters (Posten, 1994). Depending on the purpose, different models for the same bioprocess can be used and methodologies for setting up models and for selecting the suitable structure among different candidates are of interest.

6.1.1. Model structure characterisation techniques

One of the most critical steps in the model development process is the structure characterisation of the models (see Chapter 1). Structure characterisation consists of defining the adequate level of complexity of the model required as well as of determining the relationships between variables. It can be considered as the selection of the best model structure among different candidates (Dochain and Vanrolleghem, 2001).

There are different techniques and approaches for model structure characterisation covering different scientific areas. This step of the model building process is not as developed as some of the other steps (as e.g. parameter estimation). None of the existing approaches and techniques appear to be superior and clearly recommendable over others (Akaike, 1974; Marino *et al.*, 1992; Spriet and Herman, 1983). The available techniques for model structure characterisation can be classified in terms of their impact on the time needed for the whole process of both model selection plus parameter estimation.

Most of the methods evaluate the quality of a certain model structure by fitting the model to experimental data, these methods are termed as *a posteriori* methods for structure characterisation (Dochain and Vanrolleghem, 2001) and require a previous parameter estimation to fit simulation to data. Methods for structure characterisation not requiring a previous parameter estimation and capable of selecting a model structure, are called *a priori* methods. *A priori* structure characterisation can be thus defined as model selection based on the analysis of preliminary data. There are many techniques, more or less generally applicable, reported in the literature but the field is considered still underdeveloped (Ljung, 1999).

The importance of an adequate structure characterisation is even higher when dealing with models of biological systems, where the iterations through the modelling loop frequently require structure modifications (Vansteekiste and Spriet, 1982). The peculiarities of systems involving living beings, characterised by higher levels of uncertainty, non linear behaviour and large variability, make good methods for structure characterisation in these systems of much more interest (Dochain and Vanrolleghem, 2001).

Modelling of wastewater treatment systems is often controlled by the limited understanding of the underlying biochemistry and microbial interactions. Simplified schemes of the reality to enable the mathematical treatise and simulation of the resulting models are used. The development of these models can lead to an increasing complexity by adding more and more biological information to the model structure.

Particularly, modelling of anaerobic bioprocesses has remained active in the last years, trying to implement the new mechanistic knowledge about the bioconversions involved in these processes (Mosey, 1983; Costello *et al.*, 1991; Bernard *et al.*, 2001; Angelidaki *et al.*, 1993; Siegrist *et al.*, 1993; Vavilin *et al.*, 1994). Much of these knowledge was integrated in the development of the IWA Anaerobic Digestion Model No.1 (ADM1) that is a general purpose model with a considerable level of complexity in terms of number of processes and parameters (Batstone *et al.*, 2002). The assumptions made in anaerobic digestion models and particularly in the ADM1 are based mainly on assuming conversions with fixed stoichiometry and specific kinetic models. This seems to be inherited from the early models of wastewater treatment processes that have applied these principles successfully to many applications, mainly involving aerobic processes. The anaerobic ecosystems present however some peculiarities and different characteristics that question the suitability of these modelling approaches.

During recent years much development and application of the ADM1 has been reported (Batstone *et al.*, 2005) and some weak points identified. The high complexity of the model for certain applications is an issue of major concern and a technique for assessing the optimum level of complexity for these kinds of models acquires a potential great interest.

6.1.2. General mass balance

The dynamic behaviour of bioprocesses in stirred tank bioreactors is often represented by a general mass balance model for the liquid phase (Bastin and Dochain, 1990) (see Eq. 6.1)

$$\frac{dx}{dt} = D\cdot(x_{in} - x) - Q(x) + K\cdot r(x) \qquad \text{Eq. 6.1}$$

where $x = (x_1\ x_2\ \dots\ x_n)^T$ is the (n x 1) vector of measurable concentrations of the species in the liquid; D is the dilution rate; Q represents the loss of mass of each specie by transfer to the gas phase; $r = (r_1\ r_2\ \dots\ r_p)^T$ is the (p x 1) vector of conversion rates considered and K is the (n x p) matrix containing the stoichiometry of all these conversions.

This general representation assumes an underlying network of chemical and biological reactions whose stoichiometry is represented in the columns of the matrix K. The higher is the number of reactions considered, the higher is the number of columns of matrix K.

The choice of a reaction network and its stoichiometry matrix associated is generally the result of modelling assumptions. Several combinations are often possible among reaction networks of different complexity. A problem arises however, when it is intended to reduce the network complexity into a simpler model to better adapt it to the amount and quality of experimental information available (Bernard *et al.*, 2005).

When the on-line concentrations of the species are measured but the structure of the reaction network, and therefore the matrix K, are questionable, a methodology to determine the size of the reaction network from the available data would be very valuable. The choice of the size of matrix K is given by the number of independent relevant reactions that are distinguishable from the available experimental data. A methodology for assessing the dimension of matrix K is here presented and applied to several case studies in order to infer a practical method for both model structure characterisation and model reduction purposes. The minimum number of reactions describing the variability of the process will be determined without prior knowledge of the underlying network.

In this work a methodology based on principal component analysis (PCA) is presented for assessing the dimension of the system, i.e. the number of relevant processes, by using only the experimental data available and without need of any prior parameter estimation.

The work aims at developing directly applicable techniques for both structure characterisation and model reduction by using the PCA-based methodology. This provides a practical tool to enable the model objectives to be the decisor about the most convenient level of complexity in anaerobic bioprocess models.

6.2. Methodology to assess the system dimension

The general mass balance expression from Eq. 6.1 can be expressed according to Eq. 6.2 and integrated according to Eq. 6.3.

$$dx - \left[D \cdot (x_{in} - x) - Q(x)\right] dt = K \cdot r(\cdot) dt \qquad \text{Eq. 6.2}$$

$$x_{(t)} - x_{(t_0)} - \int_{t_0}^{t} \left[D \cdot (x_{in} - x) - Q(x)\right] dt = \int_{t_0}^{t} K \cdot r(\cdot) dt \qquad \text{Eq. 6.3}$$

In order to determine the minimum number of reactions (i.e. the dimension of matrix K) it will be assumed that the vector of species concentrations and inflow/outflow are measured over the time and present significant variations with time. The number of variables measured is also assumed to be larger than the number of reactions ($n > p$) while the matrix K and the vector of reaction kinetics r are unknown.

The left side term of Eq. 6.3 is independent from the reaction kinetics and can be computed from experimental data. For each species for which a mass balance is set up according to Eq. 6.1, the expression from Eq. 6.3 can be computed in a general way, no matter if they are liquid or gas phase species. Thus in the case of liquid dissolved species (as VFAs) the mass balance is computed at each time instant from the concentrations (x and x_{in}) (in e.g. mol/L_{liq}) and the dilution rate (D) (in e.g. h^{-1}) while the term Q remains zero. For the case of gas phase species (e.g. methane) the mass balance is computed analogous with null liquid concentrations and being the Q term (in e.g. mol/L_{liq}·h) who contributes to the balance. Thus for each time instant a value for the left side term of Eq. 6.3 can be computed.

Since noise is normally present in experimental data, they are filtered by using a moving average filter of size T. After filtration, $u(t)$ and $w(t)$ are defined according to Eq. 6.4 and Eq. 6.5.

$$u(t) = x_{(t)} - x_{(t-T)} - \int_{t-T}^{t} \left[D \cdot (x_{in} - x) - Q(x) \right] dt \qquad \text{Eq. 6.4}$$

$$w(t) = \int_{t-T}^{t} r(\cdot) dt \qquad \text{Eq. 6.5}$$

and thus Eq. 6.3 can be rephrased as Eq. 6.6 for a constant stoichiometry matrix K.

$$u(t) = K \cdot w(t) \qquad \text{Eq. 6.6}$$

Finally in order to remove the effect of magnitude or units used for each different species, the data are normalised. Thus the system variability can be assessed in terms of more comparable variables.

The question of assessing the dimension of matrix K can be now formulated as the assessment of the dimension of the image of K or the dimension of the space where $u(t)$ lives. Matrix K must be assumed to be also a full rank matrix, otherwise it would mean that the same dynamic behaviour could be obtained with a matrix of lower dimension (Bernard and Bastin, 2005). The determination of the dimension of the $u(t)$ space is a classical problem in statistical analysis and corresponds to the principal component analysis (PCA). It determines the dimension of the vector space spanned by the vector k_i that correspond to the rows of K (Johnson and Wichern, 1992).

For a set of *N* records of $u(t)$ the (*n* x *N*) matrix $U = [u(t_1)\ u(t_2)\ \ldots\ u(t_N)]$ is considered. The unknown associated matrix of reaction rates $W = [w(t_1)\ w(t_2)\ \ldots\ w(t_N)]$ is assumed full rank i.e. that none of the reaction rates is a linear combination of others. More time instants t_i than state variables are required as well (*N* > *n*). Given these assumptions it can be proven that the *n* x *n* matrix $M = U \cdot U^T = K \cdot W \cdot W^T \cdot K^T$ has rank *p*. Since M is symmetric it can also be written as $M = P^T \cdot \Sigma \cdot P$ where P is an orthogonal matrix ($P^T \cdot P = I$) and

$$\Sigma = \begin{pmatrix} \sigma_1 & 0 & & & \cdots & & 0 \\ 0 & \sigma_2 & 0 & & & & 0 \\ \vdots & 0 & \ddots & & & & \\ & & & \sigma_p & & & \\ & & & & 0 & & \vdots \\ & & & & & \ddots & \\ 0 & & \cdots & & \cdots & & 0 \end{pmatrix}$$

with $\sigma_{i-1} \geq \sigma_i > 0$ for $i \in \{2,...,p\}$ (Bernard *et al.*, 2005)

This is a direct application of the singular decomposition theorem since rank(M) = rank($K \cdot M$) = rank (K) = rank (Σ) = *p*. Thus the number of reactions can be determined by counting the number of non zero singular values of $U \cdot U^T$.

The reaction network sought is an approximation of the real chemical and metabolic network that can be of much larger complexity and dimension. With this scheme the fast and slow reactions will appear as perturbations of the dominant much lower dimension reaction network sought (Bernard *et al.*, 2005).

In practice perturbations of the ideal system presented above must be considered. The measurements will appear corrupted by noise especially in biological systems, frequently lacking reliable on-line sensors under the typical operation conditions. The numerical implementation of the filter to compute $u(t)$ requires interpolations (linear interpolation is used) to estimate values at the same time instants for x, and additional perturbations are thus generated.

The integration of Eq. 6.4 is done numerically different for t < *T* and for t >= *T*. Integration between 0 and t for t < *T* and between t-*T* and t for t >= *T*. Note that for large values of *T* respect to the experiment duration, the conditions at t = 0 have also a larger effect, since the integration will be for most of the points between 0 and t (since t<*T* for most of the points). The size of *T* must be chosen considering both the more or less fast dynamics wanted to be described and the level of noise demanding filtering of the data.

Large values of *T* mean that long term dynamics are addressed and are less interesting for control purposes. They give also too much weight to the initial conditions when *T* is a large percentage of the total time of the experiment. Too small values of *T* provide however almost no filtering to the data and the additional variability due to noise leads to a higher number of reactions returned to describe a given percentage of variability. The presence of noise in the data will typically cause a higher dependence of the variability described on the *T* value. Regular increases in the percentage of variability described vs. increasing number of reactions considered are a typical indicator of this noise-related extra variability.

The functions of the percentage of variability described versus the number of reactions considered are obtained by the *Matlab* function *pcacov*. This function returns the principal components or new axis to represent the system showing the maximum variability, the variances and the percentage of variability described by each principal component.

6.3. Structure characterisation from experimental data

The methodology proposed has been applied to several experimental data sets from two types of reactors and wastewater at different levels of perturbation of the system. The percentages of variability described, depending on the number of reactions for different values of the time window *T* (see Eq. 6.4), are obtained. They provide information about the number of relevant processes taking place. The assessment of the number of reactions together with an eventual subsequent structure formulation, constitute an *a priori* structure characterisation methodology.

6.3.1. Pilot scale anaerobic digester treating ethanol

In order to determine the number of relevant processes in a pilot scale UASB-AF reactor treating diluted wine, the data obtained from several overload experiments are used. The characteristics of the wastewater, measurement devices, anaerobic reactor and monitoring and control system have been described in detail in Chapter 4 of this thesis.

i) Single increase and decrease of influent organic load

A first case study consists of a single load increase and decrease on the system with on-line and off-line measurements from several state variables. Considering the information available, the variability of this system is studied in terms of these variables and mass balances are set up for ethanol, propionate, acetate, carbon dioxide (as total inorganic carbon), hydrogen, methane and total organic carbon, according to Eq. 6.4. The species in gas phase are assumed to have a null concentration in the liquid. Figure 6.1 presents an overview of the experiment, showing the operational conditions and some variables.

The percentage of variability of the system described, as a function of the number of reactions, is obtained by application of the PCA-based methodology described above. After proper set up of the mass balances (see Eq. 6.1) the data are filtered using a moving window of size *T* (see Eq. 6.4) and the PCA decomposition applied.

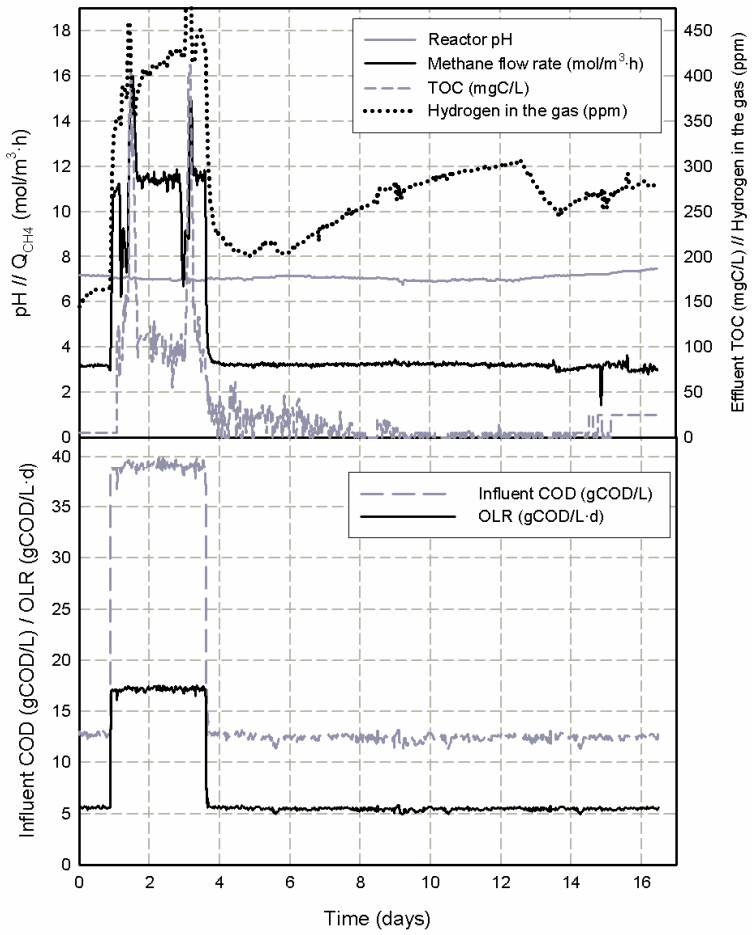

Figure 6.1. Experimental results of a single increase and decrease of influent organic load in a pilot scale UASB-AF reactor treating diluted wine.

The process is repeated for different values of the time window *T*. The results obtained are presented in Figure 6.2. Between 90-95 % of the variability of the system could be described by considering only 4 reactions at any time scale larger than 15 min. Reasonable good description of the system could be achieved with 3 reactions for larger *T* values. Long term descriptions (large *T* values) have an unclear meaning with too much weight given to

the initial conditions if *T* is a large percentage of the total time. Also, assuming that the value of the time window *T* is related to the dynamic time scale level being described, these results are of very low interest for control purposes.

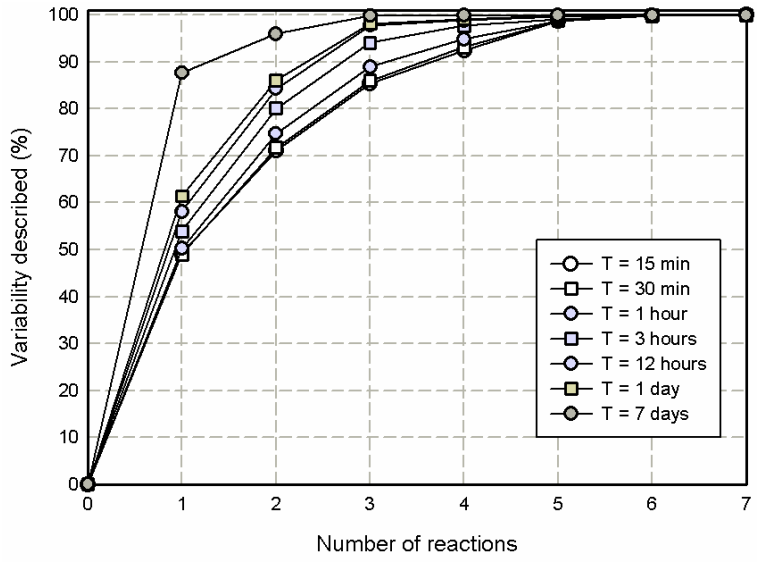

Figure 6.2. System variability versus number of processes considered for different time windows of the data filter. Single increase and decrease of load in an UASB-AF reactor.

ii) Series of increasing steps of organic loading rate

A second case study consists of a set of increasing organic loading rates causing a final system overload with a complete inhibition of methanogenesis. The anaerobic reactor is perturbed in this experiment in a wide range, starting at a pseudo steady state normal operation with moderate load and subjected then to a series of increasing overloads until its maximum resistance. Mass balances are set up for seven variables namely ethanol, butyrate, propionate, acetate, hydrogen, carbon dioxide, methane and total organic carbon, and the variability of the system studied in terms of these variables. An overview of the experiment is presented in Figure 6.3 with the influent concentration and organic loading rate applied as well as key variables showing the response of the system.

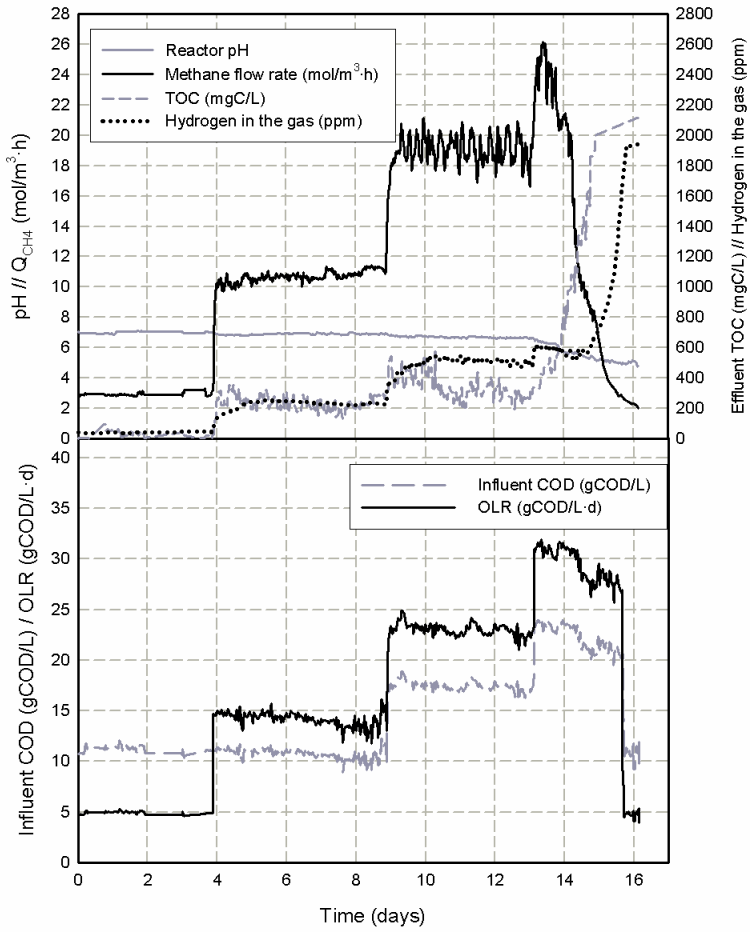

Figure 6.3. Experimental results of a series of increasing steps in organic loading rate in a pilot scale UASB-AF reactor treating diluted wine.

The same PCA methodology is applied to assess the system dimension using different time window values for the filter (see Figure 6.4). Note that since the total organic carbon is almost equivalent to the sum of the three volatile fatty acids considered in a linear dependence, 100% of the variance is retained with seven reactions. Only seven really independent variables are present.

The results show how by considering 4 reactions, more than 90 % of the system variability is already described for any time window and more than 95 % with 5 reactions. These results are of special relevance because, while they correspond also to a real experiment with presence of noise in the data, in this case the system is strongly perturbed in a wider range of different levels, including a complete inhibition of methanogenesis.

Like for the previous experiment, the number of processes required for a certain percentage of variability appeared to be considerably dependent on the size of the time window *T* used. This, together with the more regular increases in the variability described, is consequence of the presence of noise in the data at low and medium *T* values. The functions of variability obtained for long term (i.e. large *T* values) are of limited interest for control purposes and particularly for the *T* value of 7 days due to the too strong effect of the initial conditions with *T* of almost half of the experiment duration.

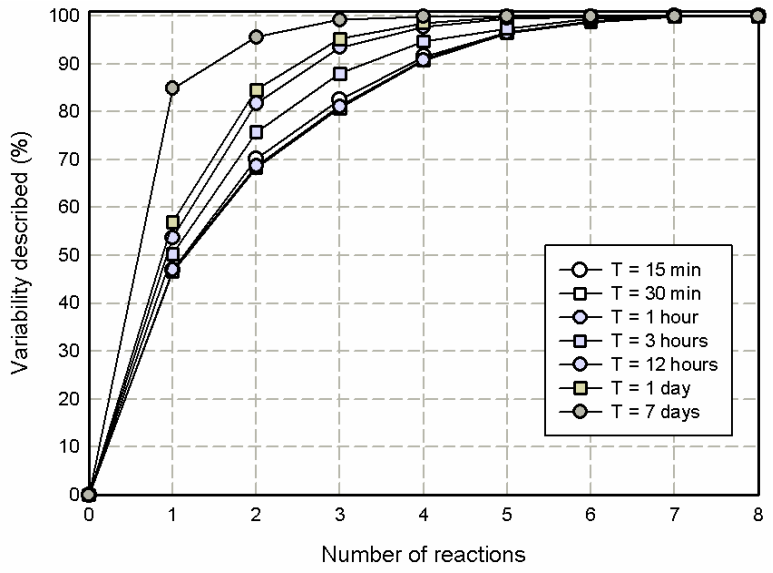

Figure 6.4. System variability versus number of processes considered for different time windows of the data filter. Series of increasing load rates in an UASB-AF treating ethanol.

iii) Perturbation of organic load on a close loop controlled system

Apart from the overload experiments above, additional data have been obtained by connecting to the anaerobic reactor a close loop controller based on the measurement of hydrogen (Rodríguez *et al.*, 2005) and submitting the system to perturbations in the influent concentration. Under perturbation the controller tried to maintain the system under stable operation by correcting the influent flow rate.

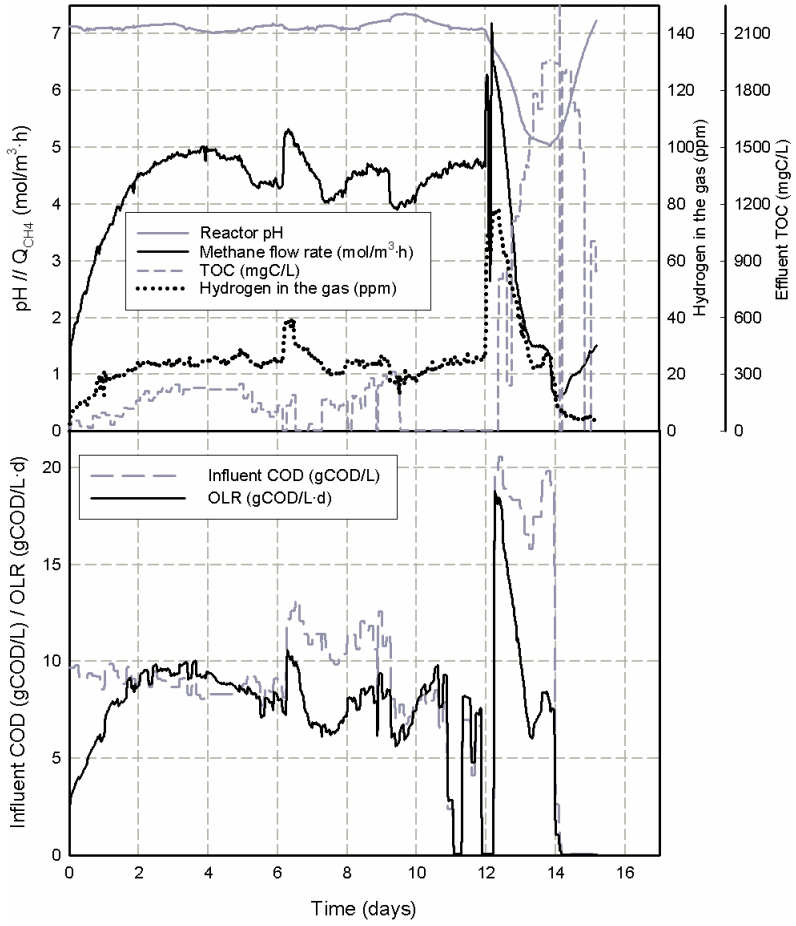

Figure 6.5. Experimental results of a controlled pilot scale UASB-AF reactor treating diluted wine under several perturbations.

During the experiment, the system was perturbed by increasing and decreasing the influent concentration under close loop control. The data generated are used for assessing the system dimension. In this experiment and considering the measurements available, the variability of the system could be studied in terms of only five variables, namely dissolved organic carbon, inorganic carbon, acetate, hydrogen and methane. An overview of the first experiment and the dynamic response is given in Figure 6.5.

The results after application of the PCA methodology for different *T* values (see Figure 6.6) show how more than 95 % of the variability of the system, as defined above, is described by considering only 4 reactions. A considerable level of noise in the data is also observed in view of the important effect of the value of *T* on the variability description.

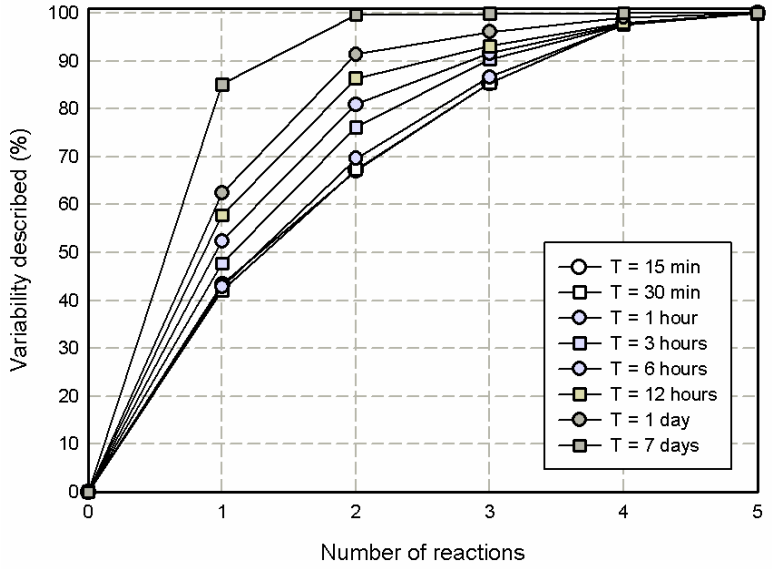

Figure 6.6. System variability versus number of processes considered for different time windows of the data filter. Controlled UASB-AF reactor under perturbations.

The results obtained for this experiment with the system under close loop control are influenced both by the low number of measured data available and by an important presence of noise in the measured data, including also some failure events in the second experiment. The results suggest that the variability of the experiment with five variables could be described by considering 4 reactions. Despite the results of this experiment,

where only a few measured variables are available, are not conclusive, they are in agreement with the results obtained for the open loop experiments with the single increase and decrease and with the increasing steps of organic loading rate, with more measured variables available.

6.3.2. Anaerobic CSTR treating industrial wastewater

The methodology to assess the system dimension is also applied to an experimental set of data, obtained from a very long experiment in a 2 L CSTR anaerobic reactor. The reactor was fed with industrial wastewater from a distillery plant whose characteristics have been presented in Table 4.3 (Chapter 4 of this thesis). During the experiment, changes in the organic loading rate were applied. The complex nature of the wastewater used caused that only low organic loading rates could be achieved. An overview of the experiment is presented in Figure 6.7. The data sampling frequency was lower in this experiment than in those with the better instrumented pilot plant but the frequency of the off-line measurements was reasonable high especially during the transient periods.

From the available measurements mass balances are set up for seven variables namely effluent COD, total volatile fatty acids, inorganic carbon, butyrate, propionate, acetate and methane. The system variability is studied in terms of these seven variables.

The results of the PCA-methodology (see Figure 6.8) showed again that 4 reactions can describe more than 90 % of the system variability. The presence of noise in the data seems to be moderate given the moderate dependence of the variability functions from the value of T chosen. Due to the low sampling frequency, the conclusions obtained from this experiment are not of large relevance for control applications but they are again in agreement with the results obtained in the previous experiments with more frequent data sampling in a different type of reactor and wastewater.

From these group of experiments with two different types of wastewater and two reactors, the system dimension methodology showed that the anaerobic systems studied could be described by considering only 4 reactions. The wide range of experimental conditions support the hypothesis that a model incorporating only 4 reactions can achieve a satisfactory dynamic description of anaerobic processes within the ranges of these experiments.

Once the system dimension is defined using the PCA-based methodology, candidate model structures can be proposed on view of the model objectives and considering 4 reactions. The modeller's expertise together with the information contained in the PCA axis provide the basis upon which candidate model structures can be built.

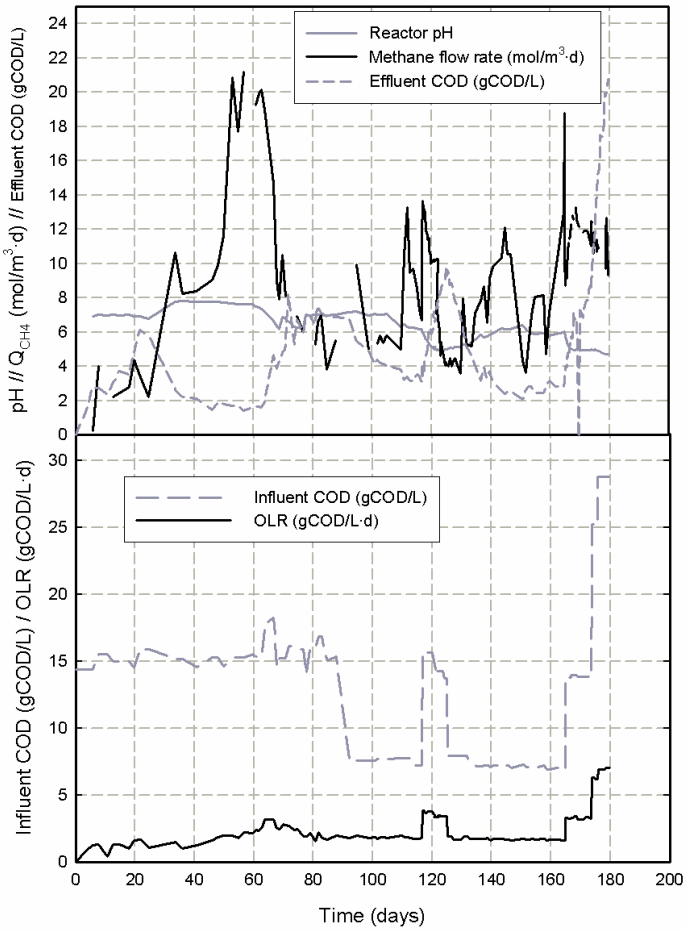

Figure 6.7. Experimental results of the operation of a CSTR treating industrial wastewater.

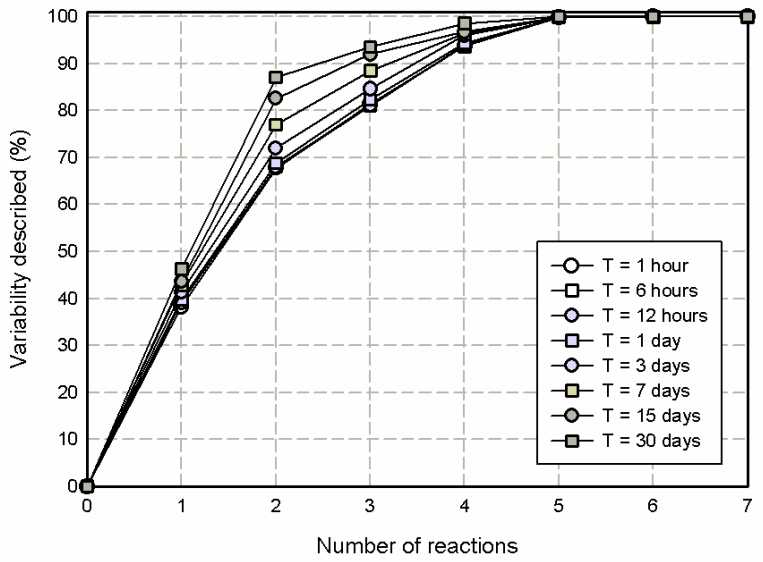

Figure 6.8. System variability versus number of processes considered for different time windows of the data filter. CSTR reactor treating industrial wastewater.

6.4. Model reduction technique from simulated data

The system dimension methodology has been above applied to experimental data as an *a priori* structural characterisation technique (Dochain and Vanrolleghem, 2001). The methodology can be also used for simplification of existing models with the same approach. In order to describe a certain set of experimental conditions without having to completely identify all the parameters of a complex existing model, a model reduction algorithm is of great interest.

A model reduction algorithm could be proposed starting with a first simulation of an experiment with the complex model to be simplified and using rough parameter estimations. By application of the PCA-based methodology for system dimension assessment, the number of relevant processes to describe the variability of the simulation data is obtained and with this information a simplified model structure can be proposed. The simplified model will contain less number of processes and therefore less number of parameters to be identified.

To illustrate part of the methodology proposed, simulations of one of the UASB-AF experiments and of the CSTR experiment presented above, are conducted with the modified version of the ADM1 (see Chapter 4 of this thesis) and with the standard ADM1 (Batstone *et al.*, 2002) respectively. When assessing the model dimension using the PCA methodology, a proposal for model reduction can be derived for the intended application. The simulation data generated must therefore correspond to similar experimental conditions and magnitude of system perturbation than the real system at which the reduced model is being aimed.

i) Simulation of the series of increasing steps of OLR in the UASB-AF pilot scale reactor

The modified version of the ADM1 incorporating an ethanol degradation process, as described in Chapter 4, is used for the simulation of the series of increasing steps of organic loading rate in the pilot scale UASB-AF reactor. An overview the simulation results obtained is presented in Figure 6.9. The system variability is defined in terms of the same seven variables as for the experimental case, namely ethanol, butyrate, propionate, acetate, hydrogen, carbon dioxide and methane.

The results of application of the PCA system dimension assessment method to the simulated data are shown in Figure 6.10. Unlike the results obtained with experimental data, a much lower dependence of the variability functions respect to the size of the filter time window T is observed. The comparison of this dependence for the simulation case, with absence of noise in the data, with the experimental case, shows the effect of the data noise on the variability functions at different T values.

The results showed how more than 90 % of the variability of the system is described by considering only 3 reactions and an almost fully described with only 4 reactions. However, the number of reactions recommended for this case is 4 considering that simulations generate very clean data and a higher variability description can be demanded.

The setup of a simplified model, considering only 4 processes, is the next step of the model reduction procedure. The definition of these processes is a difficult task and they could be derived by combination of the PCA results obtained and the modeller's expertise.

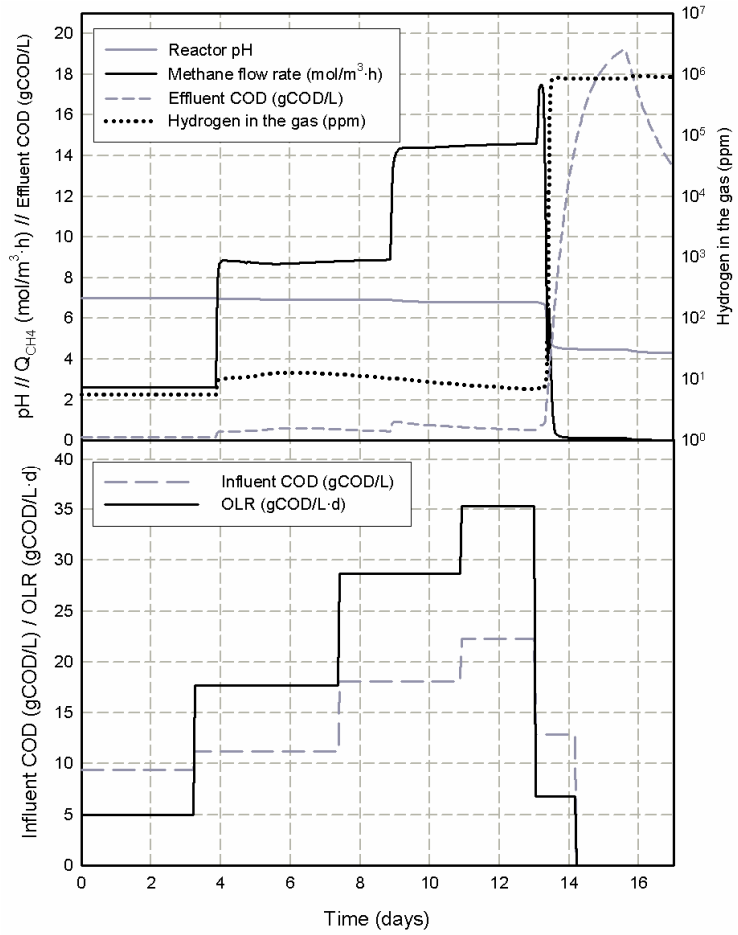

Figure 6.9. Simulation results with the modified ADM1 of several increasing steps of OLR in a pilot scale UASB-AF reactor treating diluted wine.

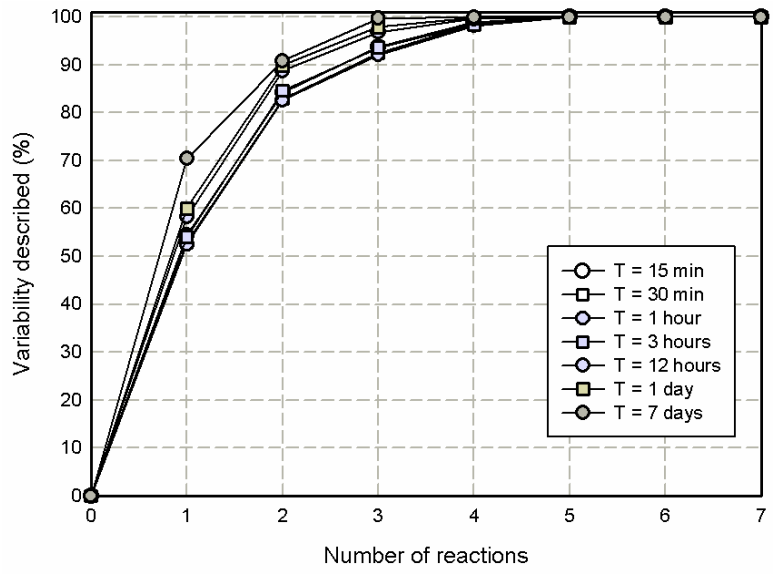

Figure 6.10. System variability versus number of processes considered for different time windows of the data filter. ADM1 simulation of an UASB-AF reactor treating diluted wine.

ii) Simulation of the anaerobic CSTR treating industrial wastewater

The standard ADM1 (Batstone *et al.*, 2002) is used to simulate the experiment with the CSTR reactor treating industrial wastewater from a distillery plant. An overview of the simulation results is presented in Figure 6.11. In this case more variables are used for the system variability definition namely soluble COD, total volatile fatty acids, inorganic carbon, butyrate, propionate and acetate, methane and hydrogen and total COD in the reactor. The system variability is studied in terms of these 9 variables. The results of application of the PCA methodology to the simulated variables (see Figure 6.12) show an almost complete independence of the variability functions respect to the value of *T*. The cleanliness of the simulation data causes that the filter has almost no effect in the results.

Very similar results than for the UASB-AF reactor simulation are found, only 3 reactions describe more than 90 % of the system variability and 4 reactions provide almost a complete description of the system. Again, considering the cleanliness of the simulation data, a number of 4 reactions is recommended for a reduced model structure.

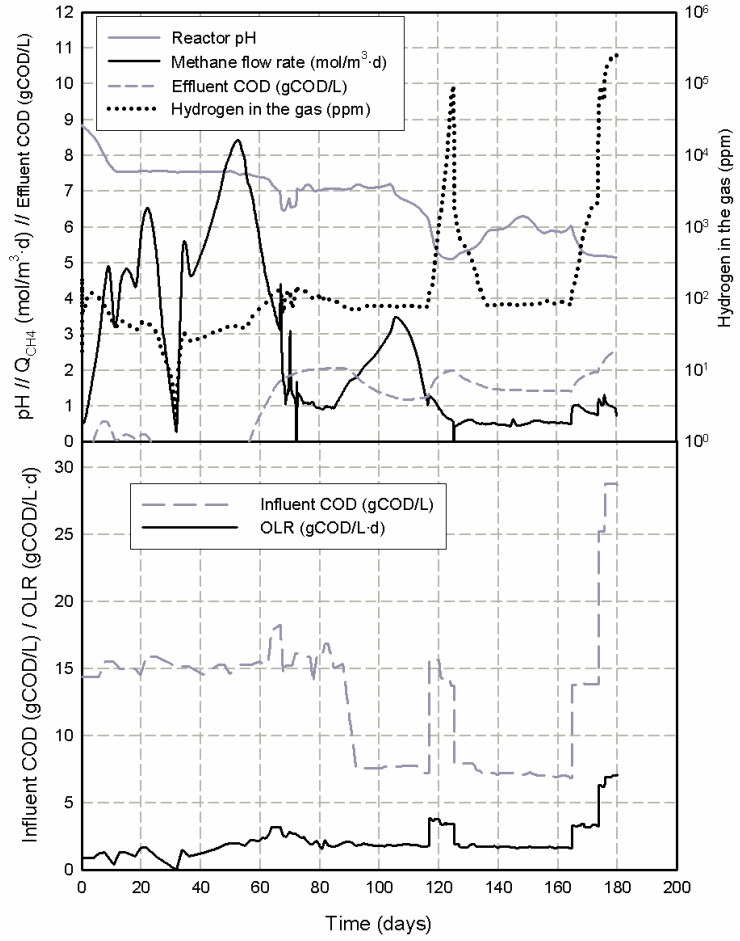

Figure 6.11. ADM1 simulation results of the operation of a CSTR treating industrial wastewater.

In addition to the dimension assessment of the simulated CSTR with 9 variables, it was performed also considering the same 7 variables than for the experimental data and the results obtained (see Figure 6.13) are equivalent and lead to the same conclusions, 4 reactions are recommended for adequate description of the system. The correlation existing between some variables causes the very similar results obtained for both cases.

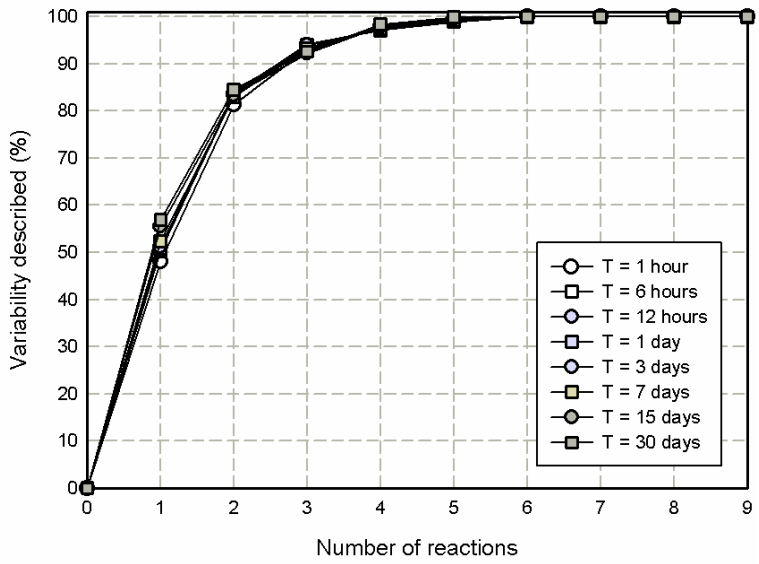

Figure 6.12. System variability versus number of processes considered for different time windows of the data filter. ADM1 simulation of a CSTR treating industrial wastewater (I).

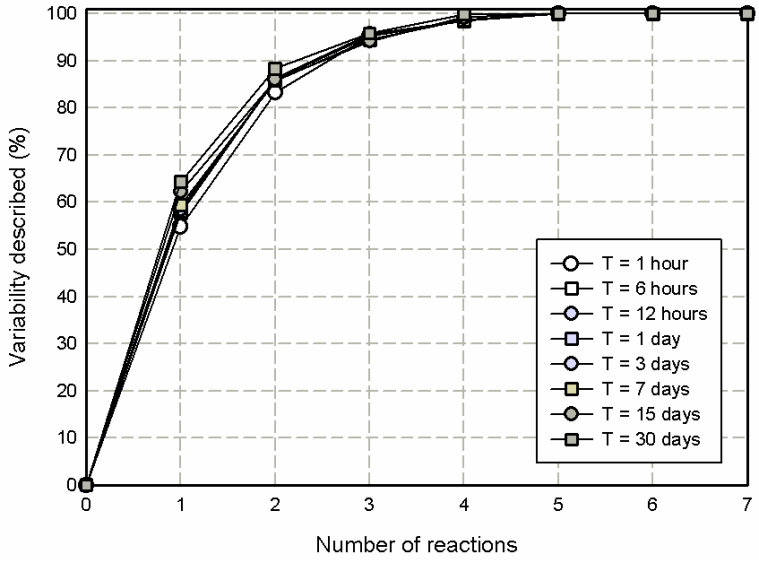

Figure 6.13. System variability versus number of processes considered for different time windows of the data filter. ADM1 simulation of a CSTR treating industrial wastewater (II).

The results obtained for the simulations of both the UASB-AF and the CSTR experiments led to the same conclusion. A reduced model considering 4 processes could provide a very good description of the simulated systems. This reduced model would behave equivalent to the complex models under the experimental conditions of the simulations.

The results obtained agree partially with other authors (Bernard *et al.*, 2005) when concluding that more simple model structures can describe the variability of anaerobic digestion processes. However depending on which and how many variables are chosen for description, the number of reactions recommended here is 4 and less reactions (Bernard *et al.*, 2005) are considered not enough to achieve a good description of the system under a reasonable wide range of operational situations, as the cases studied here. The number of 4 processes recommended offers a reasonable balance between model complexity and variability described. Proposition of a 4 processes model by an expert modeller could achieve good description of the anaerobic digestion systems while the number of parameters derived remains acceptable.

6.5. Discussion and conclusions

The application of the PCA-based methodology for system dimension assessment presented here appeared to be a powerful tool for two different purposes through the model building process, namely as *a priori* structure characterisation and as model reduction technique.

For the first case the PCA-based methodology provides very useful information for structure characterisation of models using only experimental data and with no need of previous parameter calibration for selection among candidate models. The application of the PCA-based methodology to experimental data provides information about the number of processes that should be incorporated into a candidate model to describe the variability showed in the experiments.

Once the number of relevant processes is known, the modeller must set up the most suitable processes for a good candidate model structure. This is a particularly difficult task for which some useful information can be extracted from the PCA output.

In this work the PCA-based methodology is applied to experimental data obtained from two different anaerobic reactors and with two types of wastewater. In view of the measurements available, the variability of the experiments is defined by setting up all possible mass balances according to Eq. 6.4 to apply de PCA. The results obtained showed that, for all cases studied, by considering only 4 reactions the experimental variability is described in more than a 90 %. This result is considered relevant because of the large amount of data available and different experimental conditions under which they had been obtained. This strongly supports that this number of processes is enough to describe the anaerobic digestion dynamics within a wide range of operational conditions, under the fixed stoichiometry (constant matrix K) approach.

Through the model building process, one of the key steps is the structure characterisation of the model (see Chapter 1). The methodology used here provides a powerful tool to propose candidate model structures, based on experimental information and making use not only of the explicit information of the data but also of the latent implicit information present in the dynamic data. A model structure characterisation methodology is proposed (see Figure 6.14, left side).

The PCA-based technique can be used also to assist in the definition of the processes to include in the model, based on the information provided by the principal components obtained (PCA axis). The axis interpretation is however not straightforward because their information content is not explicit. Particularly the PCA axis returned are linear combinations of the measured state variables and they are related to the stoichiometry of the conversion processes to be considered. The modeller's expertise and knowledge about the process is crucial to find the proper processes. There are also methodologies available to help with this problem (Bernard and Bastin, 2004).

A second application of the PCA-based methodology is for model reduction. Given a model of large complexity with many parameters to be identified (as it is the case of ADM1), a model reduction for structure simplification leads to a model easier to identify and validate. A new simplified model structure should moreover not decrease significantly the capacity of the model to describe experimental data within the experimental range studied.

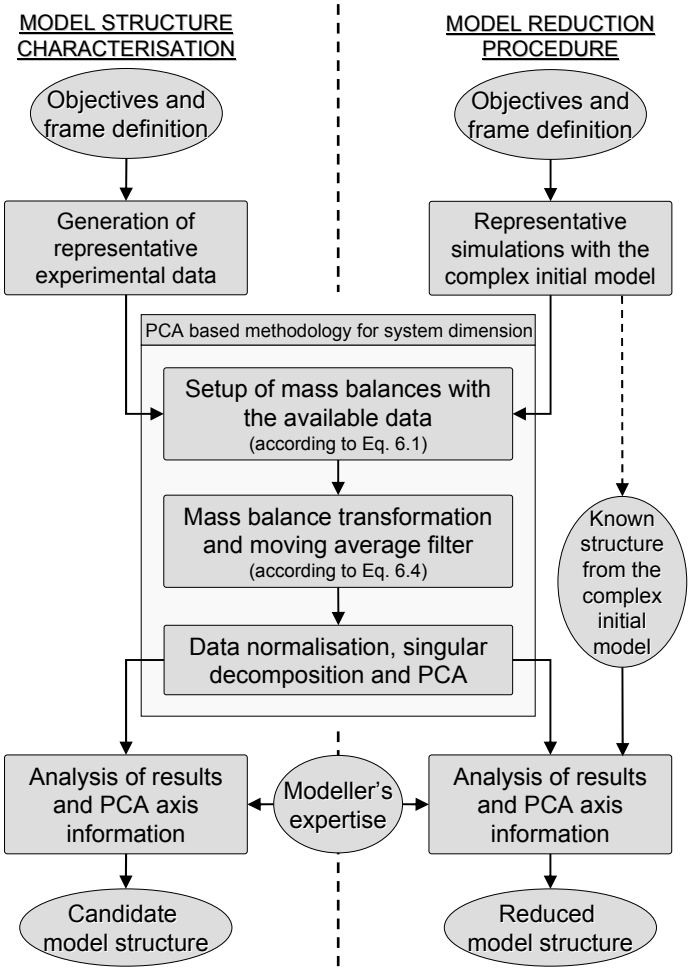

Figure 6.14. Methodologies for model structure characterisation (left side) and model reduction (right side) proposed, using the PCA-based system dimension assessment.

The PCA-based methodology is applied to simulation data using the standard and a modified version of the ADM1. The simulations reproduced experiments with the UASB-AF and with the CSTR reactor. After application of the methodology, the results show that 4 reactions are enough to describe almost totally the variability of the two anaerobic systems simulated. From a model reduction perspective this means that the complex ADM1 model could be simplified into a four-processes model with no significant loss of description of the data for the experimental cases studied.

Again, to achieve this model reduction, a simplified model structure must be derived considering the information provided by the PCA axis obtained. As for the structure characterisation method, this is a difficult task and the modeller's experience is crucial. In this case however, the information of the existing processes from the original complex model provides a starting point to infer the best process stoichiometries for the reduced model. Thus, the model reduction method (see Figure 6.14, right side) shares the PCA-based methodology with the structure characterisation method. The PCA-based system dimension assessment methodology can be applied for these two modelling purposes in general and not only for anaerobic digestion processes.

The results obtained concerning the anaerobic digestion process show that the systems studied treating hydrolysed substrates can be described by 4 independent processes, if a fixed stoichiometry is assumed, according to Eq. 6.1. An alternative interpretation is however possible for the results if variable stoichiometry is considered. Under a variable stoichiometry approach the method is not applicable at this stage because Eq. 6.6 is not valid with a variable matrix K. The recent modelling approach using variable stoichiometry (see Chapter 5 of this thesis and Rodríguez *et al.*, 2006) can be assisted by the PCA methodology to assess at which level a conversion in the system must be included as variable stoichiometry or as a new process with fixed stoichiometry.

6.6. References

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GENERAL CONCLUSIONS
CONCLUSIONES GENERALES
CONCLUSIÓNS XERAIS

General conclusions

This thesis contributes to the modelling of anaerobic mixed cultures fermentation (MCF) processes from three different perspectives. In the first section (Chapter 2 and Chapter 3) a novel approach for modelling is proposed. The second section deals with specific applications and extensions of the general purpose IWA Anaerobic Digestion Model No.1 (Chapter 4 and Chapter 5). Finally, a third section presents a statistic technique for model reduction and structural characterisation (Chapter 6). The following general conclusions are drawn on these three major topics of this thesis:

I) Metabolic network and energetic control approach for modelling anaerobic MCFs.

1. Generalized biochemical pathways of anaerobic fermentations can be used to derive a steady state model for description of glucose fermentation to a range of different products in a mixed culture of microorganisms. The model framework allows for redox and elemental balancing and for thermodynamic state analysis.

2. The new metabolic network approach presented for modelling product formation in MCF processes is solved by application of an optimal energetic efficiency criterion. The MCF model developed is able to predict the steady state metabolic product composition as a function of the environmental conditions imposed on the mixed culture like pH, hydrogen and substrate concentrations. Herewith this is the first mechanistic model that relates product formation to environmental conditions in mixed culture fermentations.

3. The MCF model predicts a change from volatile fatty acids production to ethanol production at low pH values. This is partly due to major membrane potential dissipation as a result of back-diffusion of the fatty acids into the cell at low pH values. Furthermore, the model predicts a change in dominant product formed from acetate to butyrate at increasing hydrogen partial pressure and increasing substrate concentrations. This is the result of thermodynamic limitations in the acetate production pathways at high acetate, and/or hydrogen concentrations. Experimental information under well controlled conditions is necessary for conceptual validation and further improvement of the MCF model developed.

4. Several important limitations of the initial MCF model developed have been identified and need to be addressed. This includes the necessity for inclusion of electron carriers other than NAD, the possible importance of intermediate formate formation, the

introduction of coenzyme A as conserved moiety, etc. Many of the limitations are a result of the rigidity of the assumptions made (as true steady state, thermodynamic control or selective pressure by energy efficiency) and of biochemical and physiological uncertainties. Other limitations are related to computational issues associated with the solution of the global optimisation problem required to simulate the model.

II) Implementation, extension and improvement of the Anaerobic Digestion Model No.1.

1. The implementation of the Anaerobic Digestion Model No.1 (ADM1) in molar units, instead of the initially proposed COD-based units, provides a clearer basis for stoichiometric interpretation of the bioconversions and an easier close of mass balances. Algebraic implementation of the physicochemical system in ADM1, including pH calculations, is preferred over dynamic implementation.

2. The ADM1 presents particular difficulties to identify biomass yields independently from maximum uptake rates. The estimation of kinetic parameters must be accompanied by a discussion of the assumptions regarding the biomass yield.

3. Despite the noticeable interest of the ADM1, the limitations recently reported, as complex influent characterisation and large number of parameters required, make its application recommended preferably for dynamic situations like the development and tuning of controllers.

4. The modified ADM1 proposed, incorporating ethanol degradation processes, extends the applicability of the model to ethanolic wastewater. The extended model with the new model parameters, estimated from experimental information, reproduces properly the dynamics of a wide range of experimental conditions.

5. The fixed stoichiometry approach used in the ADM1 and modifications is a severe limitation to predict the product formation in non methanogenic fermentations. A strong effect is found however on the products of the acidogenic reactor in two step anaerobic digestion. Despite the incorporation of a variable stoichiometry in ADM1 for the conversion of glucose had no major effect on the performance predicted for methanogenic systems, its implementation is required to enable the application of the model to acidogenic systems.

III) Model structure characterisation and reduction using principal component analysis.

1. Application of a methodology based on principal component analysis (PCA) to experimental data, obtained in dynamic situations, is a powerful tool to determine the optimum level of complexity of a model aiming at the description of similar experimental conditions. This is achieved by assessing the minimum number of conversion processes required to reproduce the system dynamics.

2. The application of PCA to experimental data from the operation of a CSTR and a UASB reactors treating industrial and synthetic wastewater, indicates that no more than four relevant processes are required to adequately reproduce the dynamics of the anaerobic digestion of soluble substrates like carbohydrates or ethanol.

3. The application of the same PCA technique to simulation data, generated by a complex model, determines the number of relevant processes on the simulated system dynamics and provides very useful information for model reduction or simplification.

4. Although the PCA based technique does not indicate directly which processes must be considered in the candidate or in the reduced model, the principal components obtained are linear combinations of these processes. Modeller's expertise together with the PCA information is required to set up properly the model structures.

Conclusiones generales

Esta Tesis contribuye al modelado de los procesos de fermentación anaerobios en cultivos mixtos (FCM) desde tres perspectivas diferentes. En la primera sección (Capítulo 2 y Capítulo 3) se propone un nuevo enfoque de modelado. La segunda sección se ocupa de la aplicación específica y extensión del Modelo de Digestión Anaerobia No.1 de la IWA (Capítulo 4 y Capítulo 5). Finalmente en una tercera sección se presenta una técnica estadística para la reducción y caracterización estructural de modelos (Capítulo 6). De los resultados obtenidos en esta Tesis se pueden extraer las siguientes conclusiones:

I) Modelado de FCMs mediante red metabólica y control energético.

1. La aplicación de las rutas bioquímicas generalizadas de fermentación anaerobia permite derivar un modelo en estado estacionario que describe la fermentación de glucosa a diversos de productos en un cultivo mixto de microorganismos. Este enfoque de modelado permite un balance elemental y redox así como un análisis del estado termodinámico del sistema.

2. Este nuevo enfoque, presentado con una red metabólica para el modelado de la formación de productos en procesos de FCM, es resuelto mediante la aplicación de un criterio de eficiencia energética óptima. El modelo de FCM desarrollado es capaz de predecir la composición de productos metabólicos en estado estacionario como función de condiciones ambientales impuestas al cultivo mixto tales como el pH, hidrógeno o concentración de sustrato. Se trata del primer modelo mecanístico que relaciona la formación de productos con las condiciones ambientales en fermentaciones en cultivo mixto.

3. El modelo de FCM predice un cambio desde la producción de ácidos grasos volátiles hacia la de etanol, a valores bajos de pH. Esto se debe en parte a la mayor disipación de potencial de la membrana causada por la retrodifusión de la forma libre de los ácidos grasos hacia el interior de la célula a pH bajos en el medio. El modelo predice además un cambio desde acetato como producto dominante hacia butirato cuando aumenta la presión parcial de hidrógeno y/o la concentración de sustrato. Esto es debido a limitaciones termodinámicas en las rutas de producción de acetato a concentraciones altas del mismo y/o de hidrógeno. Para lograr la validación conceptual y mejora del modelo de FCM desarrollado, es necesaria información experimental de calidad obtenida en condiciones muy bien controladas.

4. Se han identificado diversas limitaciones importantes en el modelo inicial de FCM desarrollado que necesitan ser resultas. Entre ellas se encuentra la necesidad de incluir otros transportadores de electrones además de NAD, la importancia de la formación de

formiato como intermedio, la introducción de coenzima A como metabolito conservado, etc. Muchas de las limitaciones son resultado de la rigidez de las suposiciones asumidas (como verdadero estado estacionario, control termodinámico o presión selectiva por eficiencia energética) y de incertidumbres bioquímicas y fisiológicas. Otras limitaciones se deben a aspectos computacionales asociados con la solución del problema de optimización global requerida para simular el modelo.

II) Implementación, extensión y mejora del Modelo de Digestión Anaerobia No.1.

1. La implementación del Modelo de Digestión Anaerobia No.1 (ADM1) en unidades molares, en lugar de en unidades de DQO inicialmente propuestas, proporciona una base más clara para la interpretación de la estequiometría de las bioconversiones y un cierre más fácil de los balances de masa. La implementación algebraica del sistema de fisicoquímica en ADM1, incluyendo los cálculos de pH, es preferida frente a la implementación dinámica.

2. El ADM1 presenta una especial dificultad para la identificación de los rendimientos de biomasa independientemente de las máximas velocidades de consumo. Cualquier estimación de parámetros cinéticos debe ir acompañada de una adecuada discusión acerca de las suposiciones referentes a los rendimientos de biomasa.

3. A pesar del notable interés del ADM1, las limitaciones recientemente reportadas, como la compleja caracterización del influente y el gran número de parámetros requeridos, hace su aplicación recomendable preferiblemente para situaciones dinámicas como pueden ser el desarrollo y ajuste de controladores.

4. La modificación del ADM1 propuesta, incorporando el proceso de degradación de etanol, extiende la aplicabilidad del modelo a aguas residuales con este sustrato. El modelo extendido con los nuevos parámetros, estimados de información experimental, reproduce adecuadamente la dinámica de un amplio rango de condiciones experimentales.

5. El uso de estequiometría fija en el ADM1 y modificaciones es una severa limitación para predecir la formación de productos en sistemas no metanogénicos. Se observa sin embargo un importante efecto en la composición de los productos del reactor acidogénico en un sistema de digestión anaerobia en dos etapas. Aunque la incorporación de una estequiometría variable en el ADM1 para la conversión de glucosa no presenta efectos importantes en la predicción del funcionamiento de sistemas metanogénicos, su implementación es necesaria para aplicar el modelo a sistemas acidogénicos.

III) Caracterización estructural y reducción de modelos con componentes principales.

1. La aplicación de un método basado en el análisis de componentes principales (ACP) sobre datos experimentales obtenidos en situaciones dinámicas es una potente herramienta para determinar el nivel de complejidad óptimo de un modelo que busque la descripción de similares condiciones experimentales. Esto se logra mediante la determinación del mínimo número de procesos de conversión requerido para reproducir la dinámica del sistema.

2. La aplicación de ACP a datos experimentales procedentes de la operación de un CSTR y un reactor UASB alimentados con agua residual industrial y sintética, indica que cuatro procesos relevantes son suficientes para reproducir adecuadamente la dinámica de la digestión anaerobia de sustratos solubles como carbohidratos o etanol.

3. La aplicación de la misma técnica ACP a datos simulados, generados por un modelo complejo, determina el número de procesos relevantes en la dinámica del sistema simulado y proporciona información muy útil para la reducción y simplificación de modelos.

4. Aunque la técnica basada en ACP no indica directamente qué procesos deben considerarse en los modelos candidatos o en los reducidos, los componentes principales calculados son combinaciones lineales de esos procesos. Para plantear adecuadamente las estructuras de estos modelos es necesaria la información del ACP junto con el conocimiento experto.

Conclusións xerais

Esta Tese contribúe ó modelado dos procesos de fermentación anaerobia en cultivos mixtos (FCM) dende tres perspectivas diferentes. Na primeira sección (Capítulo 2 e Capítulo 3) proponse un novo enfoque de modelado. A segunda sección ocúpase da aplicación específica e extensión do Modelo de Dixestión Anaerobia No.1 da IWA (Capítulo 4 e Capítulo 5). Finalmente nunha terceira sección preséntase unha técnica estatística para a reducción e caracterización estructural de modelos (Capítulo 6). Dos resultados obtidos nesta Tese pódense extraer as seguintes conclusións:

I) Modelado de FCMs mediante rede metabólica e control enerxético.

1. A aplicación das rutas bioquímicas xeralizadas de fermentación anaerobia permite derivar un modelo en estado estacionario que describe a fermentación de glucosa a diversos de productos nun cultivo mixto de microorganismos. Este enfoque de modelado permite un balance elemental e redox así como unha análise do estado termodinámico do sistema.

2. Este novo enfoque, presentado cunha rede metabólica para o modelado da formación de productos en procesos de FCM, resólvese mediante a aplicación dun criterio de óptima eficiencia enerxética. O modelo de FCM desenrolado é capaz de predecir a composición de productos metabólicos en estado estacionario como función de condicións ambientais impostas ó cultivo mixto como o pH, hidróxeno ou concentración de substrato. Trátase dun primeiro modelo mecanístico que relaciona a formación de productos coas condicións ambientais en fermentacións con cultivo mixto.

3. O modelo de FCM predi un cambio dende a producción de ácidos graxos volátiles cara a de etanol, a valores baixos de pH. Isto débese en parte á maior disipación de potencial da membrana causada pola retrodifusión da forma libre dos ácidos graxos cara o interior da célula a pH baixos no medio. O modelo predi ademais un cambio dende acetato como producto dominante cara butirato cando aumenta a presión parcial de hidróxeno e/ou a concentración de substrato. Isto é debido a limitacións termodinámicas nas rutas de producción de acetato a concentracións altas do mesmo e/ou de hidróxeno. Para acadar a validación conceptual e mellora do modelo de FCM desenrolado, é necesaria información experimental de calidade obtida en condicións moi ben controladas.

4. Diversas limitacións importantes foron identificadas no modelo inicial de FCM desenrolado que necesitan ser resoltas. Entre elas atópase a necesidade de incluír outros transportadores de electróns ademais de NAD, a importancia da formación de formiato

como intermedio, a introducción de coencima A como metabolito conservado, etc. Moitas das limitacións son resultado da rixidez das suposicións asumidas (como un estado estacionario verdadeiro, control termodinámico ou presión selectiva por eficiencia enerxética) e de incertidumes bioquímicas e fisiolóxicas. Outras limitacións débense a aspectos computacionais asociados coa solución do problema de optimización global requirida para simular o modelo.

II) Implantación, extensión e mellora do Modelo de Dixestión Anaerobia No.1.

1. A implantación do Modelo de Dixestión Anaerobia No.1 (ADM1) en unidades molares, en lugar de en unidades de DQO inicialmente propostas, proporciona unha base máis clara para a interpretación da estequiometría das bioconversións e un peche máis sinxelo dos balances de masa. A implantación alxebraica do sistema de fisicoquímica en ADM1, incluíndo os cálculos de pH, é preferida fronte á implantación dinámica.

2. O ADM1 presenta unha especial dificultade para a identificación dos rendementos de biomasa independentemente das máximas velocidades de consumo. Calquera estimación de parámetros cinéticos debe ir acompañada dunha adecuada discusión acerca das suposicións referentes ós rendementos de biomasa.

3. A pesares do notable interese do ADM1, as limitacións recentemente reportadas, como a complexa caracterización do influínte e o grande número de parámetros requiridos, fai a súa aplicación recomendable preferiblemente para situacións dinámicas como poden ser o desenvolvemento e axuste de controladores.

4. A modificación do ADM1 proposta, incorporando o proceso de degradación de etanol, estende a aplicabilidade do modelo a augas residuais con este substrato. O modelo estendido cos novos parámetros, estimados a partires de información experimental, reproduce adecuadamente a dinámica dun amplo rango de condicións experimentais.

5. O uso de estequiometría fixa no ADM1 e modificacións é unha severa limitación para predecir a formación de productos en sistemas non metanoxénicos. Obsérvase nembargantes un importante efecto na composición dos productos do reactor acidoxénico nun sistema de dixestión anaerobia en dúas etapas. Aínda que a incorporación dunha estequiometría variable no ADM1 para a conversión de glucosa non presenta efectos importantes na predicción do funcionamento de sistemas metanoxénicos, a súa implantación é necesaria para aplicar o modelo a sistemas acidoxénicos.

III) Caracterización estructural e reducción de modelos con compoñentes principais.

1. A aplicación dun método baseado na análise de compoñentes principais (ACP) sobre datos experimentais obtidos en situacións dinámicas é unha potente ferramenta para determinar o nivel de complexidade óptimo dun modelo que busque a descrición de similares condicións experimentais. Isto acádase mediante a determinación do mínimo número de procesos de conversión requirido para reproducir a dinámica do sistema.

2. A aplicación de ACP a datos experimentais procedentes da operación dun CSTR e dun reactor UASB alimentados con auga residual industrial e sintética, indica que catro procesos relevantes son suficientes para reproducir adecuadamente a dinámica da dixestión anaerobia de substratos solubles como carbohidratos ou etanol.

3. A aplicación da mesma técnica ACP a datos simulados, xerados por un modelo complexo, determina o número de procesos relevantes na dinámica do sistema simulado e proporciona información moi útil para a reducción e simplificación de modelos.

4. Aínda que a técnica baseada en ACP non indica directamente qué procesos deben considerarse nos modelos candidatos ou nos reducidos, os compoñentes principais calculados son combinacións lineais destes procesos. Para prantexar adecuadamente as estructuras destes modelos é necesaria a información da ACP xunto con coñecemento experto.

P.1. Journal papers

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P.3.1. Oral papers

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