Effect of culture temperature on the heterologous expression of *Pleurotus eryngii* versatile peroxidase in *Aspergillus* hosts

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Abstract

Production of recombinant versatile peroxidase in *Aspergillus* hosts was optimized through the modification of temperature during bioreactor cultivations. To further this purpose, the cDNA encoding a versatile peroxidase of *Pleurotus eryngii* was expressed under control of the alcohol dehydrogenase (alcA) promoter of *Aspergillus nidulans*. A dependence of recombinant peroxidase production on cultivation temperature was found. Lowering the culture temperature from 28ºC to 19°C enhanced the level of active peroxidase 5.8-fold and reduced the effective proteolytic activity 2-fold. Thus, a maximum peroxidase activity of 466 U L-1 was reached. The same optimization scheme was applied to a recombinant *Aspergillus niger* that bore the alcohol dehydrogenase regulator (alcR), enabling transformation with the peroxidase cDNA under the same alcA promoter. However, with this strain, the peroxidase activity was not improved, while the effective proteolytic activity was increased between 3- and 11-fold compared to that obtained with *A. nidulans*.

Keywords: *Aspergillus*, proteases, temperature, versatile peroxidase

1. Introduction

Ligninolytic enzymes have been extensively investigated because of their biotechnological potential for lignin removal in paper pulp manufacturing and in the degradation of aromatic xenobiotics [1, 2, 3]. Two ligninolytic peroxidases, lignin peroxidase (LiP) [4, 5] and manganese peroxidase (MnP) [6], were described in *Phanerochaete chrysosporium* and reported in other white-rot fungi from the group of basidiomycetes [7, 8]. A third ligninolytic peroxidase, characterized by its ability to oxidize both Mn^{2+} and different aromatic compounds and dyes - including those that are not efficiently oxidized by LiP and MnP, has been reported in *Pleurotus* and *Bjerkandera* species [9, 10, 11]. The genes encoding two isoenzymes of this new versatile peroxidase (VP) have been cloned from *Pleurotus eryngii*: *vpl*, expressed in peptone-containing liquid cultures as two allelic variants; and *vps1*, expressed during lignocellulose solid-state fermentation [12, 13]. *P. eryngii vpl* has been expressed in the model ascomycete *Emericella nidulans* (conidial state *Aspergillus nidulans*) under control of the *alcA* promoter [14]. *A. nidulans* is a genetically well-characterized fungus compared with industrial species such as the deuteromycete-type *Aspergillus niger* and *Aspergillus oryzae.* However, higher levels of recombinant proteins can be obtained from these industrial species [15].

Heterologous expression of active ligninolytic peroxidases from *P. chrysosporium* has been reported in baculovirus systems [16], *Escherichia coli* [17, 18], *Pichia pastoris* [19], *A. oryzae* [20], *A. niger* [21, 22], and *A. nidulans* [23]. However, in no cases have the ligninolytic peroxidase levels been satisfactory, taking into account the amount of enzyme required for its biotechnological application. This low yield has been attributed, among other reasons, to factors related to the hemoprotein nature of the enzyme, operating at the post-translational level [24]. Some efforts are being directed at better understanding the importance of this aspect of heterologous peroxidase production through the application of genetic engineering techniques [24, 25]. On the other hand, only a few studies have been reported that deal with improving the production of these recombinant enzymes through the optimization of operational conditions.

Both optimization approaches - genetic or operational control improvements - require the identification of the expression bottleneck as a first step. Bioreactor cultivation, in which operational parameters can be easily monitored and controlled, is a very useful tool in identifying such problems. The magnitude of the problem and the feasibility of the optimization of culture conditions may determine whether genetic or process engineering techniques should be used. The optimization of culture conditions seems to be an adequate alternative for improving heterologous peroxidase production, because it is possible to obtain an improvement of the heterologous protein expression by simply optimizing a process variable [26]. However, if a cause-effect relationship could be postulated, this knowledge can be used to improve the heterologous expression by a further modification of the recombinant system. In the present work, the influence of temperature in bioreactor cultivations is considered as a means of improving the production of recombinant *P. eryngii* VP (VP*) in two *Aspergillus* hosts.

2. Materials and methods

2.1 Chemicals

Hemin and threonine (>99%) were obtained from Fluka. Bovine serum albumine (BSA, fraction V) was obtained from Merck. All the components used in growth and induction media were obtained from Sigma-Aldrich.

2.2 Organisms and culture media

The recombinant *A. nidulans* (*bi*A1, *meth*G1, *arg*B2) strain (IJFM A742) harboring the *P. eryngii vpl* cDNA (encoding the allelic variant VPL2) under control of the *alcA* promoter [14] was grown in complete medium, [27] supplemented with D-biotin (10 μg L^{-1}) and L-methionine (74.5 mg L^{-1}). The recombinant *A. niger* (gpd*alc*R, *csp*A1, *leu*A1, *nic*A1, *pyr*A6) strain bearing the *alc*A regulator (*alc*R) of *A. nidulans* was cotransformed according to the method described by Punt et al. [28] with the same construction used for *A. nidulans* transformation and the vector PAN7-1, which contains the hygromycin B resistance marker from *Escherichia coli* under the *A. nidulans gpd* gene

promoter [29]. The recombinant *A. niger* strain harboring the *vpl* cDNA was grown in glucose-peptone medium [30] supplemented with leucine (250 mg L^{-1}) and nicotinic acid (1 mg L^{-1}).

Asexual spores (conidia) were removed from Petri dishes by adding 5 mL of sterilized water after a 48 h growth period at 37ºC. 10 mL of the spore solution were used to inoculate 1 L of growth medium for experiments with *A. nidulans*. In all other experiments, the initial spore concentration of the growth medium (typically 1 x 10^8 spores mL^{-1}) was fixed by first determining the concentration of the spore solution using a Bürker-Türk counting chamber. Both *Aspergilli* were grown for a period of 24 h at 28°C and 180 rpm in 1-L Erlenmeyer flasks containing 200 mL of culture medium (growth phase). The washed mycelium was then transferred to the induction medium, with the same composition but containing 0.05% glucose (subinhibitory concentration), 0.1 M threonine (as an inducer), and 0.05% hemin (induction phase). The addition of hemin was required for peroxidase production [14]. Subsequent cultivation of induced cultures was carried out in a bioreactor (as described below).

2.3 Bioreactor cultivation

Bioreactor cultivation was carried out in a BIOSTAT-Q ® fermentation system (B.Braun Biotech International, Melsungen, Germany) equipped with four 1-L vessels. pH was kept constant at 6.8 and 5.5 during *A. nidulans* and *A. niger* cultivation, respectively, by the addition of 2 M NaOH or H_2SO_4 using an automatic control system. The other operational conditions were temperature 19°C (unless otherwise stated), air flow (2 vvm), and agitation rate (500 rpm, agitated by magnetic stirrers). The initial working volume of all experiments was 0.5 L. When simultaneous experiments were conducted with the same organism, the different vessels were inoculated from a common growth culture. The culture with *A. nidulans* at the optimal temperature was conducted in triplicate in independent fermentations. In all other cases, the cultures were carried out once. Bioreactor cultures of *A. niger* gp20' lacking active copies of the *vpl* gene was inoculated with spore solutions (1x10^8 spores mL^{-1}). All other conditions were similar to those described above.

2.4 Enzymatic activities

VP activity in culture filtrates was estimated from formation of the Mn^{3+}-tartrate complex (λ_{238}, 6500 M^{-1} cm^{-1}) during oxidation of 0.1 mM $MnSO_4$ in 0.1 M sodium tartrate, pH 5.0, in the presence of 0.1 mM H_2O_2 [9]. Control measurements without H_2O_2 were taken. One unit of VP activity was defined as the amount of enzyme oxidizing 1 µmol of Mn^{2+} per minute.

Extracellular protease activity was determined according to the method of van den Hombergh et al. [31]. A 450 µL diluted sample was incubated with 50 µL of 1% (w/v) BSA in 0.1 M sodium acetate buffer (pH 4 and 6.8, for *A. niger* and *A. nidulans* samples, respectively) at 37ºC. After 60 min, reactions were stopped with 500 µL of 10% (w/v) trichloroacetic acid (TCA). Then, the sample and a non-incubated control were kept at 0°C for 30 min, the precipitated proteins were removed by centrifugation at 6,000 rpm for 5 min, and the optical density of the TCA-soluble fractions was measured at 280 nm. Before calculating protease units, control absorbance was subtracted from sample absorbance. One unit of protease activity was defined as the amount of enzyme causing a change of one absorbance unit per hour.

3. Results

3.1 Bioreactor cultivation of recombinant A. nidulans

Cultivations of *Aspergillus nidulans* have traditionally been performed in the 28ºC to 37ºC range [32]. However, it has been shown that, in many cases, the optimum growth conditions are not necessarily the best for the expression of a heterologous enzyme [33]. Previous observations had shown hardly detectable VP activity in shaken cultures of recombinant *A. nidulans* without temperature control, although the temperatures attained (up to 32ºC) did not reduce fungal growth. However, when the temperature was maintained constant at 28°C, consistent VP activity around 50 U L^{-1} resulted [26]. In the present work, in order to investigate the effect of temperature on VP* production, five discontinuous batch bioreactor cultures were conducted at different temperatures (31, 28, 25, 19 and 16°C), using complete medium [14] (Fig. 1). The highest activity was obtained

at 19°C (466 U L^{-1}), while the lowest VP activity was obtained at 31°C (24 U L^{-1}). A correlation between maximal VP activity and the time needed to reach that activity was found (shown in Fig. 1). Thus, the highest and lowest VP activity peaks were obtained after 115 and 14 h, during the fermentations conducted at 19 and 31ºC, respectively.

It can be observed that the temperature effect on VP activity over the experimental range is not symmetrical with respect to that which is optimum for active VP* production. VP activity slowly decreased when the temperature was increased from 19ºC to 25ºC, while a sharp activity drop was produced by decreasing temperature from 19ºC to 16ºC. When independent fermentations at the optimal temperature were repeated in triplicate, a standard deviation of 9.4% of the maximal VP activity was found.

When extracellular protease activity was measured in samples taken from the cultures conducted at the different temperatures, a similar pattern between VP* production and activity of extracellular proteases was observed (Fig. 2). The maximal level of both MnP and proteolytic activities were reached at the same time, and this time was progressively delayed at lower temperatures.

3.2 Bioreactor cultivation of recombinant A. niger

A. niger was chosen for the expression of VP*, since high levels of recombinant proteins can be obtained from this host organism [15]. Since *A. niger* and *A. nidulans* are related species, similar fermentation conditions could be applied. Moreover, the use of a recombinant *A. niger* strain (bearing *A. nidulans alc*R) enabled VP* expression under the same *alc*A promoter used in *A. nidulans*. Thus, the next objective was to apply the optimization scheme found for *A. nidulans* to improve the expression of VP* using *A. niger* as the host organism.

Two simultaneous bioreactor cultures with a recombinant *A. niger* strain harboring the *vpl* cDNA, were conducted at a) the optimal temperature found for the expression of the vpl2 peroxidase in *A. nidulans* (19ºC) and b) the optimal growth temperature of *Aspergillus* (28ºC) (Fig.

3). The highest VP activity was obtained at 19°C (412 U L^{-1}), while at 28°C only 26% of this activity was found (107 U L^{-1}). An inverse correlation between VP activity and extracellular protease level at the two temperatures was observed. This tendency is similar to that found with *A. nidulans* (Fig. 2), but the protease levels were much higher in the recombinant *A. niger* cultures. A third bioreactor was inoculated with *A. niger* spores from a strain lacking VP* production, which resulted in the absence of extracellular proteases. Since extracellular proteases can strongly affect the production of active VP*, protease production in both *Aspergillus* hosts was investigated in more detail.

3.3 Effect of temperature on proteolytic activity of culture broths of A. niger and A. nidulans.

The effect of temperature on extracellular protease activity was investigated using filtered culture broths from bioreactor fermentations of *A. nidulans* and *A. niger* (after 7 and 11 days, respectively), each concentrated 30-fold by ultrafiltration (10-kDa cut-off membrane). The protease activity of the concentrated culture broths from *A. nidulans* and *A. niger* fermentations measured at 19ºC were 6132 and 5040 U/L, respectively. Extracellular protease activity was analyzed at 37, 31, 28, 25 and 19°C. In Fig. 4 it can be observed that the activity of the proteolytic enzymes is significantly reduced by lowering the incubation temperature. Thus, *A. nidulans* protease activities at 28 and 19°C are 38% and 9% of that measured at 37°C, respectively; while for *A. niger* they are 40% and 19%, respectively. Since routine extracellular protease assays were performed at 37°C, the actual values of the above results were taken into account to compare the effective proteolytic capacities of *A. nidulans* and *A. niger* cultures grown at different temperatures (Table 1). The lower the temperature of the culture, the lower the production of proteases in both *Aspergillus* strains, this difference being higher for *A. niger* cultures. It is important to highlight that the time necessary to reach the maximal VP activity was double for *A. niger* cultures.

4. Discussion

Operational control during bioreactor cultivation can greatly improve the production of recombinant proteins. In a previous work, we had demonstrated that the production of *P. eryngii* VP* in *A. nidulans* is considerably reduced as the pH of the medium increases under pH-free conditions [26]. By simply keeping the culture pH constant during bioreactor cultivation, it was possible to improve active VP* 5.3-fold. The culture temperature used in those experiments was 28ºC, which is considered as the optimal growth temperature of *Aspergillus* sp. In the present study, we studied the effect of culture temperature on VP* production using *A. nidulans* and *A. niger* as host organisms. As in previous studies, the *vpl* cDNA encoding a VP isoenzyme expressed in liquid cultures of *P. eryngii* was used for heterologous expression [14].

4.1 Effect of temperature on VP production*

Bioreactor cultures at different temperatures demonstrated that a small increase of temperature from 28ºC drastically reduced the production of active VP*, while active VP* production was increased 5.8-fold by reducing the temperature from 28 to 19ºC. The effect of culture temperature on heterologous expression has been observed in other recombinant systems [34, 35, 36]. Improvement of laccase expression in *Saccharomyces cerevisiae* and of interleukin-3 in *Streptomyces lividans* have previously been obtained by lowering temperature [33, 37]. The opposite effect of temperature has also been reported [38]. However, the present paper is, to the best of our knowledge, the first report on improvement of heterologous expression of a ligninolytic peroxidase by lowering culture temperature.

The effect of temperature on heterologous gene expression has been related to: a) protein aggregation [39, 40]; b) incorrect gene translation due to the presence of rarely used codons [41]; and c) proteolytic degradation [37]. Ligninolytic peroxidases expressed in *Aspergillus* hosts are secreted into the culture medium [14, 20, 22], and no aggregation has been reported. Moreover, codons rarely used by *A. nidulans* (codon usage data from GenBank Release 127.0) were not found

in the VP cDNA (GenBank accession N° AF007222). However, as discussed below, a probable relationship between active VP* production and proteolytic degradation could be established when extracellular proteases were analyzed during recombinant *Aspergillus* cultivation at different temperatures.

4.2 VP production in* Aspergillus niger

Although VP* expression was enhanced by decreasing temperature, industrial application of this enzyme (e.g. in paper pulp bleaching or dye decolorization) requires greater VP* production. For this reason, the use of other fungal hosts was considered.

Temperature dependence of VP* production was also observed during *A. niger* bioreactor cultivation. In this case, lowering the cultivation temperature from 28ºC to 19°C caused 3.8-fold increase of VP activity. However, when comparing the VP activities produced at 19°C by the recombinant *A. nidulans* and *A. niger* strains (466 and 412 U L^{-1}, respectively), no improvement was achieved by using the second fungal host. The lower VP* production seems to be due to the high level of extracellular proteases found in *A. niger* cultures. In fact, the effective proteolytic activity in the *A. niger* cultures were 11- and 3-fold those found with *A. nidulans*, when grown at 28ºC and 19°C, respectively.

The lack of extracellular proteases during bioreactor cultivation of a recombinant *A. niger* strain that does not produce VP* suggests that the proteolytic activity is induced by expression of the recombinant peroxidase. It has been shown that proteases are responsible for the degradation of many heterologous proteins [42, 43, 44]. Furthermore, in *A. nidulans*, the production of extracellular proteases is subjected to at least four regulatory mechanisms; C, N and S metabolite repression, and pH control. A fifth regulatory mechanism, induction by exogenous protein, is present in *A. niger* [45] and may also operate in *A. nidulans* under certain conditions [46]. The latter mechanism would be responsible for VP* degradation in both *A. nidulans* and *A. niger*

cultures.

The use of protease-deficient hosts has been considered for reducing the problem of proteolytic degradation of heterologous enzymes [22]. Currently, the *vpl* cDNA has been cloned in a protease-deficient *A. niger* strain. However, even using such host organisms, the problem of heterologous protein degradation was not overcome in some cases [44, 47, 48], probably due to the complexity of the *A. niger* protease system [49].

It is noteworthy that related enzymes can be expressed with very different efficiencies in the same or related expression systems. This is the case for basidiomycete peroxidases including: *Coprinus cinereus* peroxidase (CIP), that is expressed with high yields in *A. oryzae* (CIP* is commercialized by Novozymes, Denmark); *P. chrysosporium* MnP and *Caldariomyces fumago* chloroperoxidase, which can be expressed with low yields in *A. niger* [22, 24, 50] and, in the case of MnP, also in *A. oryzae* [20]; and *P. chrysosporium* LiP, which failed to be expressed in any of the above systems [51]. It has been shown that the *P. eryngii* VP is more related to LiP than MnP in both protein sequence (and molecular structure) and gene regulation [14, 52]. Therefore, the heterologous expression yields reported here, as well as in previous studies [12], are especially relevant, although they should be improved for biotechnological applications in sectors such as paper pulp bleaching and dye decolorization where high amounts of low price enzyme are required.

Conclusions

VP* production in *Aspergillus* hosts can be significantly improved by selecting the optimal culture temperature. Proteolytic degradation of recombinant proteins often represents a limiting factor for increasing heterologous expression. The results obtained suggest that extracellular proteolytic activity involved in VP* degradation during its expression in *Aspergillus* hosts can be reduced considerably by lowering the cultivation temperature.

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Tables

Table 1. Maximal effective proteolytic activities at 28 and 19ºC during *A. nidulans* and *A. niger* cultures, calculated using the factors obtained from Fig. 4. The time at which those activities were reached is also presented.

	A. nidulans		*A. niger*	
T (ºC)	28	19	28	19
Maximal protease activity (U L^{-1})	58	31	633	93
Time (h)	22	114	41	214

Legends for figures

Fig. 1 Maximal VP activity during bioreactor cultivation of *A. nidulans* at five different temperatures (the growth period required is indicated).

Fig. 2 Time course of extracellular VP and protease activities during bioreactor cultivation of *A. nidulans* at 28ºC (▲), 25ºC (●), and 19°C (■).

Fig. 3 Time course of extracellular VP and protease activities during bioreactor cultivation of *A. niger* at 19ºC (●) and 28°C (▲), as well as *A. niger* gp20' lacking active copies of the *vpl* gene (✶).

Fig. 4 Estimation of protease activity at different temperatures in samples obtained from bioreactor cultures of *A. niger* and *A. nidulans* (void bars) after 9 and 11 days, respectively (as percentages of the activity measured at 37°C).

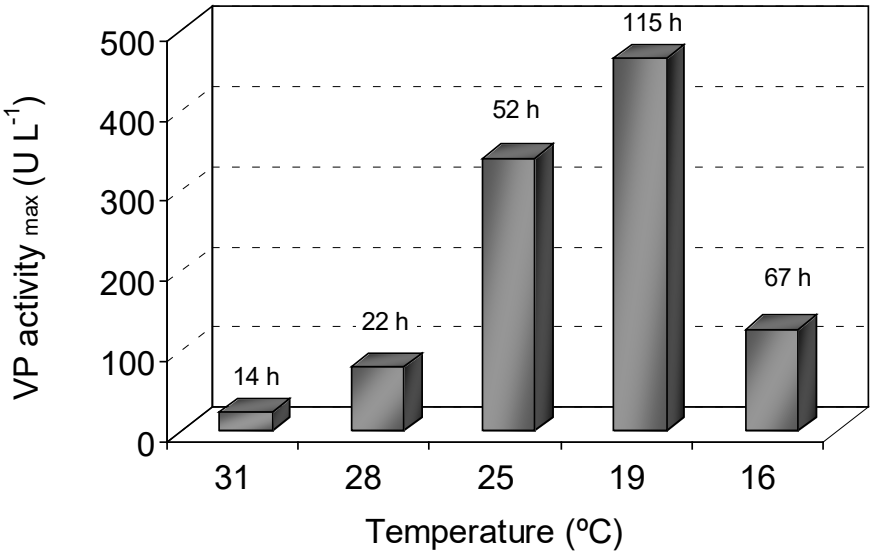
VP activity max (U L-1)
500
400
300
200
100
0
14 h
22 h
52 h
115 h
67 h
31
28
25
19
16
Temperature (ºC)

Fig. 1

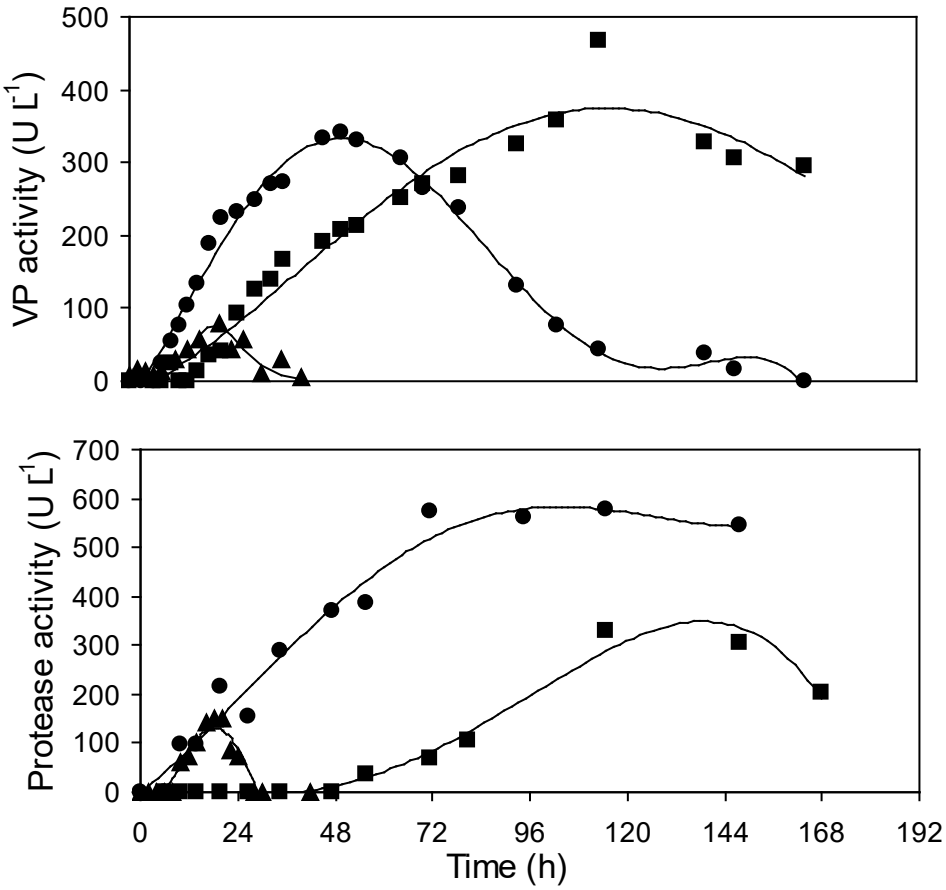
VP activity (U L-1)
500
400
300
200
100
0
Protease activity (U L-1)
700
600
500
400
300
200
100
0
0
24
48
72
96
120
144
168
192
Time (h)

Fig. 2

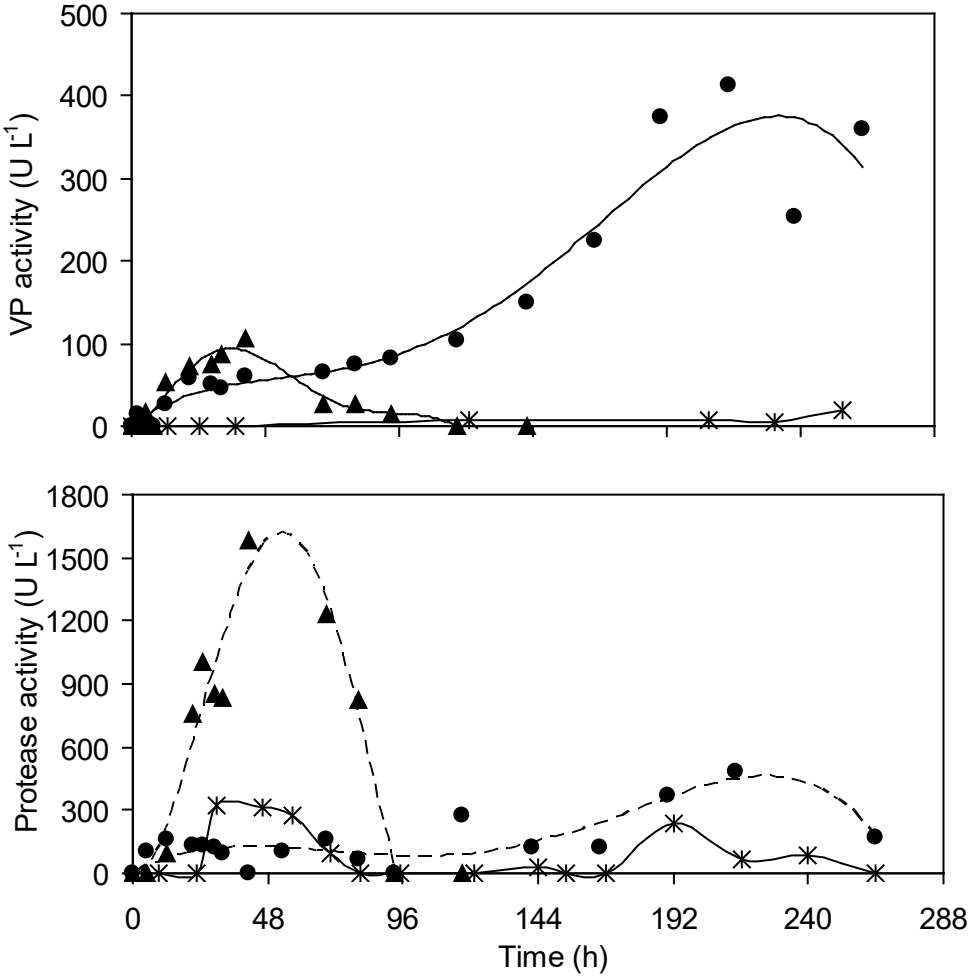
VP activity (U L-1)
500
400
300
200
100
0
Protease activity (U L-1)
1800
1500
1200
900
600
300
0
0
48
96
144
192
240
288
Time (h)

Fig. 3

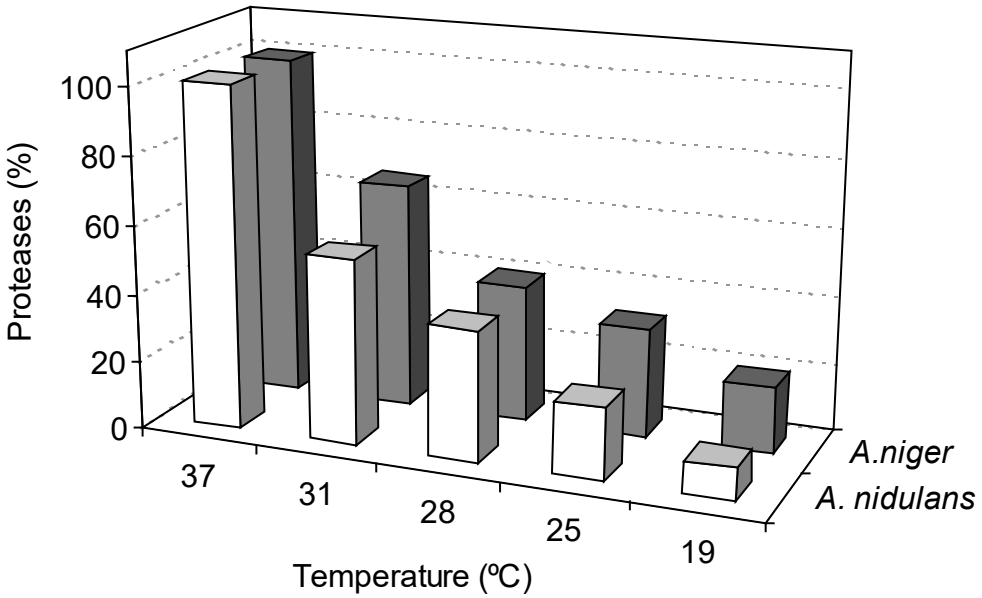
Proteases (%)
100
80
60
40
20
0
37
31
28
25
19
Temperature (ºC)
A.niger
A. nidulans

Fig. 4