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Lipase production by *Penicillium restrictum* in solid-state fermentation using babassu oil cake as substrate

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Abstract

Growth and enzyme production in SSF by a Brazilian strain of *Penicillium restrictum* was studied. Solid waste from the babassu oil industry was used as the basic nutrient source and was supplemented with peptone, olive oil or starch at different C/N ratios. The highest lipase activity (30.3 U/g initial dry weight) was achieved after 24 h of cultivation with 2% olive oil enrichment. Lipase activity was very sensitive to the kind and the level of supplementation, and decreased as protease level and pH in the media increased. Maximal levels of glucoamylase and protease were obtained with 4% starch enrichment, indicating that the type of carbon source supplemented to the basal medium determines the major enzymes produced.

Keywords: *Penicillium restrictum*; Lipase; Solid-state fermentation; Babassu cake; Amylase; Protease

1. Introduction

Lipases (EC 3.1.1.3) hydrolyse triglycerides into diglycerides, monoglycerides, glycerol and fatty acids. Interest in these enzymes has increased markedly over the last decades, as they find diverse applications as medicines (digestive enzymes), food additives (flavour-modifying enzymes), clinical reagents (glyceride-hydrolysing enzymes) and cleaners (detergent additives) [1]. Additionally, a promising application field for lipases is the biodegradation of plastics, such as polyhydroxyalkanoates (PHA) and polycaprolactone (PCL) [2,3]. New applications, such as the resolution of racemic mixtures to produce optically active compounds, should also arise from the stereospecific acting properties of some lipases [4].

Many reports of solid-state fermentation systems (SSF) have been published in recent years supporting the application of SSF in upgrading agricultural by-products and in the production of fine-chemicals and enzymes [5]. Solid state processes are therefore of special economic interest for countries with an abundance of biomass and agro-industrial residues, as these can be used as cheap raw materials.

However, only recently some reports on the production of lipase by SSF were published. Kamini et al. [6] studied lipase production from *Aspergillus niger* by SSF using gingelly oil cake as substrate. Christen et al. [7] investigated the feasibility of obtaining lipase with *Rhizopus delemar* growing on a polymeric resin. Ohnishi et al. [8] studied the production of lipase by *Aspergillus oryzae* with different solid substrates. Rao et al. [4,9] optimised the synthesis of lipase by the yeast *Candida rugosa* in SSF and found the C/N ratio of the medium to be an important parameter for lipase activity. Rivera-Muñoz et al. [10] compared the production of lipase by *Penicillium candidum* in submerged (SF) and solid state fermentations, verifying the superiority of SSF processes.

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In the present work the strain employed was a recently isolated strain of *Penicillium restrictum*, identified as a lipase producer in submerged fermentations [11]. The objective of the current study was to investigate whether this strain was also able to produce lipase under SSF conditions, growing on the agro-industrial waste from which it has been isolated. Other enzyme activities (protease and glucoamylase) were monitored to characterise more fully the growth of the fungus on the babassu cake. As lipase production in SF by this strain has been shown to be highly dependent upon the C/N ratio of the medium [12], the influence of C/N ratio of the solid medium on enzyme production was studied by enriching babassu cake with different carbon and nitrogen sources.

2. Material and methods

2.1. Microorganism

The *P. restrictum* strain employed in this work was previously isolated from the solid waste of the Brazilian babassu oil industry [11]. After cultivation for 7 days at 30°C on agar slants (soluble starch, 2%; olive oil, 2%; yeast extract, 0.1%; K_2HPO_4, 0.05%; $MgSO_4 \cdot 7H_2O$, 0.025%; $CaCO_3$, 0.5%; agar, 1%), the microorganism was maintained at 4°C.

2.2. Solid-state fermentation

The basal fermentation medium was babassu (*Orbignya oleifera*) cake, grounded and sieved to provide particle sizes between 0.21 and 0.42 mm. The composition of the babassu cake is shown in Table 1. The basal medium was enriched with peptone (1% w/w, wet basis), olive oil (1 and 2% w/w, wet basis) and starch (2 and 4% w/w, wet basis). The carbon and nitrogen contents of these substances, as determined by the Kjeldahl method [13] and total organic carbon analysis (Shimadzu, TOC Analyser 5000A), are shown in Table 2. An overview of the different media tested, their carbon and nitrogen contents and their C/N ratios are found in Table 3. The experimental set shown in Table 3 enabled a comparison of different substances enriching the basal medium at equal C/N ratios, and of the same substance enriching the basal medium at different C/N ratios. Additionally, an analysis of the effects caused by further increasing the N content of the oil cake was enabled by enriching the cake with peptone.

Table 1
Composition of the babassu oil cake

Substance	Fraction (% w/w)
Water	6.6
Protein	22.8
Lipids	4.5
Carbohydrates	61.8
Ashes	4.3

Table 2
Carbon and nitrogen content of the medium supplements

Substance	C (% w/w)	N (% w/w)
Peptone	60	11
Olive oil	88	–
Starch	44	–

The experiments were carried out aseptically in polypropylene beakers covered with microbiological filter tissue (Lamino Padding Roll, ACCO®, Tidi Products Inc.). Each beaker containing 10 g of solid substrate (dry basis) was sterilised at 111°C for 20 min and then inoculated with 10^8 spores from spore suspensions prepared in Tween 80 0.1% v/v [14]. The moisture content of the media was adjusted to 70% v/w. Cultivation was carried out at 30°C in an incubator with humidified air injection, with fermentation times varying from 15 to 65 h.

2.3. Sampling and enzyme extraction

At selected time intervals, whole beakers were taken. In each beaker the fermented solids were comminuted and mixed for achieving uniformity. Three portions of 0.5 g were then withdrawn for assessing moisture content, glucosamine content and pH. Smits et al. [5] have shown that accurate determinations of dry-matter weight and glucosamine in fermented wheat bran can be made using samples as small as 0.4 and 0.1 g, respectively.

Enzyme extraction was carried out by adding to the remainder of the fermented solids in each beaker 45 ml of 50 mM phosphate buffer (pH 7.0), and then shaking the mixture in a rotary shaker (200 min^{-1}) for 30 min at 37°C, a temperature high enough to increase the extraction efficiency, without causing enzyme denatura-

Table 3
Carbon and nitrogen contents, and C/N ratio in fermentation media

Medium	C (% w/w)	N (% w/w)	C/N ratio
Babassu cake (BC)	44.8	3.6	12.4
Cake + 1% peptone (CP)	46.8	4.0	11.7
Cake + 1% olive oil (CO-1)	47.8	3.6	13.3
Cake + 2% olive oil (CO-2)	50.7	3.6	14.1
Cake + 2% starch (CS-2)	47.8	3.6	13.3
Cake + 4% starch (CS-4)	50.7	3.6	14.1

tion [11]. The raw extract was obtained by pressing the mixture and subsequent centrifugation ($1600 \times g$, 2 min). The supernatant was used to determine enzyme activities.

2.4. Enzyme activity assays

2.4.1. Lipase

Determination of lipase activity in the raw enzyme extract followed the procedure described in previous works [11,12]. According to this procedure, 18 ml of an emulsion of arabic gum (5% w/v) and olive oil (5% w/v) (prepared in 50 mM phosphate buffer, pH 7.0) were mixed with 2 ml enzyme extract and allowed to react for 60 min at 37°C. The reaction was then stopped through the addition of 20 ml of a 1:1 acetone/ethanol mixture. After further agitation for 10 min for total extraction of fatty acids, titration was performed with 0.05 N NaOH in a pH-stat (Mettler DL 21) until end-point 11.0. Blank assays were done by adding the acetone/ethanol mixture prior to the enzyme sample. One unit of lipase activity was defined as the amount of enzyme, which produced 1 μmol of fatty acids equivalent per minute under the assay conditions.

2.4.2. Protease

Activity was determined following the method proposed by Charney and Tomarelli [15], which is based on the reaction of the enzyme extract with a 0.5% azocasein solution at 37°C and pH 5.0. One unit of proteolytic activity was defined as the quantity of enzyme that produced a unitary difference in absorbance between the reaction blank and the sample per minute under the assay conditions.

2.4.3. Glucoamylase

Activity was determined as in Ref. [16] by reacting the enzyme extract with a 4% soluble starch solution at 45°C and pH 4.2. As in the work of Pandey et al. [17], one enzyme unit was defined as the amount of enzyme, which produced 1 μmol of reducing sugars per minute under the assay conditions.

2.5. Glucosamine assay

As an indirect measurement of cell growth [17,18], glucosamine was determined according to the method proposed by Aidoo et al. [19].

2.6. Moisture content

Moisture was determined after total drying of 0.5 g of fermented material at 75°C.

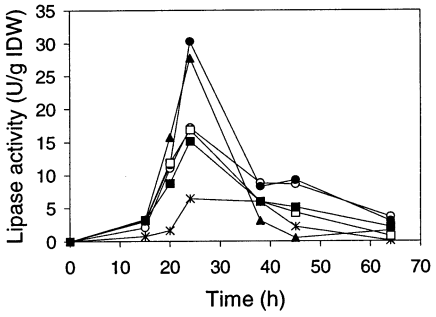

Fig. 1. Lipase activity during cultivations on babassu cake (×), cake + 1% peptone (▲), cake + 1% olive oil (○), cake + 2% olive oil (●), cake + 2% starch (□), and cake + 4% starch (■).

2.7. pH measurement

Five millilitres of distilled water were added to 0.5 g of fermented material and the mixture was agitated vigorously. After a 10-min interval for settling, the pH of the supernatant was determined with a pH-meter.

3. Results and discussion

The kinetics of lipase production in the different culture media is presented in Fig. 1. The highest amounts of lipase activity were achieved with peptone enrichment (CP medium, C/N ratio 11.7), 27.8 U/g of initial dry weight (IDW), and olive oil enrichment (CO-2 medium, C/N ratio 14.1), 30.3 U/g IDW. These results are in agreement with Ohnishi et al. [8], who affirmed that high nitrogen concentrations were effective in enhancing the production of microbial lipases, and with Rao et al. [4], who verified that oil content was a significant variable affecting the lipase yield.

For all media, the lipase peaks were obtained after short fermentation times (24 h), while in submerged fermentation this strain produced high lipase activities only after 64 h [12]. Therefore, the solid state fermentation system investigated in the present work seemed to be a promising alternative to submerged systems, as it provided high lipase productivities and uses a waste as substrate.

While supplementation with olive oil gave the best lipase results, the highest values of glucoamylase and protease activities (Figs. 2 and 3) were achieved with starch enrichment (CS-4 medium, C/N ratio 14.1), reaching values of 98.6 and 7.9 U/g IDW, respectively. This indicates that the type of carbon source used as supplementation plays a determinant role in the kind of major enzymes that will be produced by *P. restrictum*.

It is interesting to note that, for the three enzymes studied, activity maxima were obtained with media of C/N ratio 14.1. However, in the case of lipase, the N-rich CP medium gave also high activities. This is a

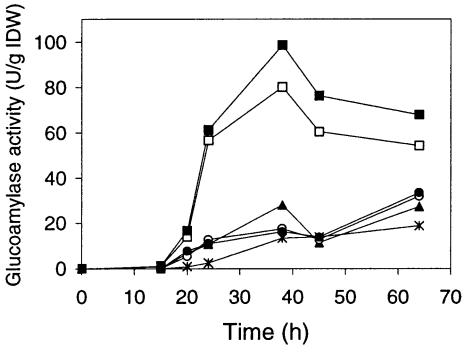

Fig. 2. Glucoamylase activity during cultivations on babassu cake (×), cake + 1% peptone (▲), cake + 1% olive oil (○), cake + 2% olive oil (●), cake + 2% starch (□), and cake + 4% starch (■).

further indication that the type of enriching substance has more influence on lipase production than the C/N ratio.

From Figs. 1–3 it is possible to note that the basal medium (BC) was able to support the production of the three hydrolases, although in a smaller amount when compared to the supplemented media. This shows that the microorganism consumed the natural substrates present in the babassu cake.

Maximum glucosamine contents achieved in the different media are showed in Table 4. The supplementation of the medium with peptone (CP medium), olive oil (CO-1 medium) and starch (CS-2 medium) caused a 35% increase in the glucosamine content when compared with the basal medium (BC medium). The supplementation with the highest amounts of olive oil and starch (C/N ratios equal to 14.1, Table 3) produced a further increase in the glucosamine content (Table 4), indicating that microbial growth was enhanced as a result of a higher availability of carbon sources.

Fig. 4 presents the highest values of the enzyme activities in connection with the different culture media and their C/N ratio. It can be observed that there was a trend for the production of the three hydrolases studied when the basal medium was enriched. Among

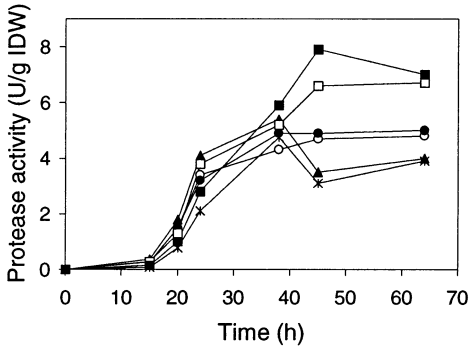

Fig. 3. Protease activity during cultivations on babassu cake (×), cake + 1% peptone (▲), cake + 1% olive oil (○), cake + 2% olive oil (●), cake + 2% starch (□), and cake + 4% starch (■).

Table 4
Maximum glucosamine content achieved in the different culture media

Medium	Glucosamine content (mg/g IDW)
Babassu cake (BC)	5.0
Cake + 1% peptone (CP)	6.7
Cake + 1% olive oil (CO-1)	6.7
Cake + 2% olive oil (CO-2)	8.0
Cake + 2% starch (CS-2)	6.7
Cake + 4% starch (CS-4)	7.1

the three enzymes studied, lipase and glucoamylase were more sensitive to medium enrichment than protease.

In the N-richest medium (CP-medium), lipase activity increased 4.3 times in relation to the basal medium, whereas the increase in protease and glucoamylase activities was only 1.2 and 1.5 times, respectively. Various authors [8,20,21] have shown that high nitrogen concentrations in culture media are effective in enhancing the production of lipase by microorganisms. Other authors [12,22] have compared different nitrogen sources for lipase production by *Penicillium* strains in SF, and have found peptone to give the best results. Therefore, the fact that enriching a basal medium already rich in proteins with peptone is effective, corroborates the statement that peptone contains certain co-factors and aminoacids, which match *P. restrictum* physiological requirements for lipase biosynthesis [12].

Culture media with the same kind of supplementation, but in different concentrations, were investigated. In the case of supplementation of the basal medium with triglycerides in increasing amounts (CO-1 and CO-2 media), there was a significant increase in lipase production—the concentration of this enzyme was 1.8 times higher in the CO-2 medium when compared with the CO-1 medium—whereas almost no modification was observed in glucoamylase and protease production (Fig. 4).

On the other hand, the supplementation of BC medium with increasing amounts of starch (CS-2 and CS-4 media) caused an increased production of glucoamylase and protease, whereas lipase production was reduced—in the CS-4 medium glucoamylase and protease production was about 1.2 times higher and lipase production was about 1.2 times lower than in the CS-2 medium (Fig. 4). Kamini et al. [6] enriched gingelly oil cake with 5% w/w starch and observed an inhibition of lipase synthesis. The reduced activity of lipase in the starch rich medium, like observed in submerged fermentations [12], may be related to the inhibitory effect of glucose, which is released by cleavage of starch.

A better understanding of the influence of C/N ratio on enzyme synthesis by this *P. restrictum* strain may be

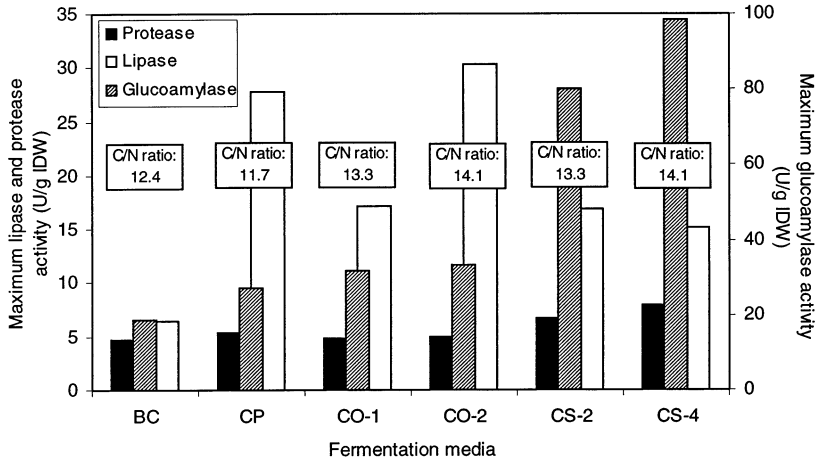

Fig. 4. Maximum enzyme activities achieved according to different C/N ratios and different supplementations.

obtained if media of different composition, but equal C/N ratio, are compared. Regarding the media with a C/N ratio of 13.3 (Fig. 4), it is observed that lipase production was almost the same in CO-1 and CS-2 media, whereas glucoamylase and protease production were 2.5 and 1.4 times higher in the CS-2 medium. However, when the media with a C/N ratio of 14.1 are compared, the increase in production of the latter enzymes was even higher—glucoamylase and protease activities were 3.0 and 1.6 times higher in the CS-4 medium than in the CO-2 medium, respectively. However, lipase production in the CS-4 medium was just 50% of the production in the CO-2 medium. These observations indicate that the type of carbon source used to enrich the basal medium has a greater influence on enzyme production than the C/N ratio.

In all fermentation media the pH started to increase after about 20 h of cultivation from an initial value of 5.0, reaching a final value of 8.0 after 65 h (Fig. 5). The peaks of lipolytic activity were reached when the pH values were between 5.0 and 6.0. Although starting with a different initial pH value, Christen et al. [7] obtained lipase peaks in a similar pH range (between 4.9 and 5.7 for their different culture media).

The gradual pH increase was probably caused by the release of proteases, which starts at about 20 h of fermentation (Fig. 3), resulting in deamination of aminoacids and liberation of ammonia. Such a pH profile was also observed in submerged fermentations [12,23].

The alkalinisation of the media, together with the accumulation of proteases, may explain the decrease in lipolytic activity after 24 h of cultivation. Previous results in submerged fermentation showed that *P. restrictum* lipase was not stable at alkaline pHs and that the decrease in lipase activity was reduced when the serine protease inhibitor (PMSF) was added at later fermentation stages [11,12]. Comparing the crude enzyme extracts to the culture broth obtained with this *P. restrictum* strain in SF [12], it is observed that protease activities in SSF are much higher than in SF. This indicates that in SSF proteolysis is a significant parameter affecting lipase production. The accumulation of proteases in SSF affecting the stability of lipases was also observed with the fungus *A. oryzae* [6] and with different species of *Penicillium* [10].

4. Conclusions

Based on the present results, it can be concluded that this strain of *P. restrictum* is able to grow and produce different hydrolases in SSF, having babassu cake as substrate. High lipase titres could be obtained using an abundant and cheap raw material. Short fermentation times (24 h) make this fermentation system a promising one, in terms of lipase productivity.

Furthermore, it was observed that supplementation of the basal medium with small amounts of carbon or nitrogen sources, leading to media with small changes in C/N ratio, produces great variations in the level of the enzyme activities obtained.

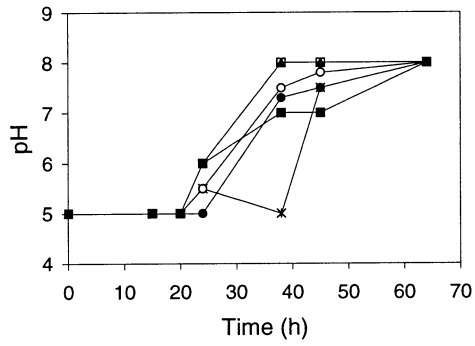

Fig. 5. pH changes during cultivations on babassu cake (×), cake + 1% peptone (▲), cake + 1% olive oil (○), cake + 2% olive oil (●), cake + 2% starch (□), and cake + 4% starch (■).

A comparison of media with different compositions, but equal C/N ratio, showed that the kind of enriching substance has a greater influence on enzyme production than the C/N ratio.

Enriching the babassu cake with different carbon sources favours the synthesis of different enzymes: olive oil supplementation results in high lipase activities, while starch supplementation results in high glucoamylase activities. Therefore, according to the application desired, the basal medium may be differentially enriched to give high yields of the desired enzyme.

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