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Lipase Production by *Penicillium restrictum* in a Bench-Scale Fermenter

Effect of Carbon and Nitrogen Nutrition, Agitation, and Aeration

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ABSTRACT

A preliminary screening work selected *Penicillium restrictum* as a promising micro-organism for lipase production. The physiological response of the fungus towards cell growth and enzyme production upon variable carbon and nitrogen nutrition, specific air flow rate (Qa) and agitation (N) was evaluated in a 5-L bench-scale fermenter. In optimized conditions for lipase production meat peptone at 2% (w/v) and olive oil at 1% (w/v) were used in a growth medium with a C/N ratio of 9.9. Higher C/N ratios favored cell growth in detriment of enzyme production. Low extracellular lipase activities were observed using glucose as carbon source suggesting glucose regulation. Final lipase accumulation of 13,000 U/L was obtained, using optimized specific air flow rate (Qa) of 0.5 vvm and an impeller speed (N) of 200 rpm. Agitation showed to be an important parameter to ensure nutrient availability in a growth medium having olive oil as carbon source.

Index Entries: Lipase; lipase production; *Penicillium restrictum*; C and N nutrition; agitation and aeration.

INTRODUCTION

There is a growing interest on microbial lipases (acylglycerol hydrolases, E.C. 3.1.1.3) that are largely used in the detergent and food industries. Lipases with peculiar catalytic properties obtained from microbial

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screening procedures may be used for the modification of fats and oils or synthesis of novel compounds that will probably have an important impact on a range of chemical industries *(1)*.

Fungi from the genera *Mucor, Aspergillus, Rhizopus,* and *Penicillium* have been used in lipase production studies. Although the effects of media composition have been described *(2–5)*, there is a general lack of discussion as to the causes of the micro-organism's physiological response. The use of triglycerides as carbon source is adequate for both cell growth and enzyme production, although the induction of lipase biosynthesis by free fatty acids or triglycerides is controversial *(6–8)*. Consistent cell growth is likewise observed when glucose is used as a carbon source although lipase production by some *Penicillium* sp, may be repressed depending on the micro-organism studied *(5)*. Concerning the nitrogen source, meat peptone is beneficial for lipase production by some *Penicillium* and other fungi species *(4,7–11)*.

The reported agitation and aeration conditions for lipase production by fungi in bench-scale fermenters varies widely: 750 rpm and 2 vvm, 200 rpm and 1.0 vvm, and 80 rpm and 1.0 vvm were used in *Geotrichum candidum (12), Humicola lanuginosa (13),* and *Rhizopus* sp *(14)* fermentations, respectively.

Aiming at a better understanding of fungi lipase production, a stepwise study was carried out, focusing on the relationship between cell growth and enzyme production in response to variable carbon and nitrogen nutrition, specific air flow rate, and agitation in a 5-L instrumented fermenter.

MATERIALS AND METHODS

Micro-organism Maintenance and Propagation

Penicillium restrictum isolated from wastes of a Brazilian babassu coconut oil and identified as lipase producer was used in the experiments. For spores production the micro-organism was cultivated in agar slants (soluble starch 2%; olive oil 1%; yeast extract 0.1%; $MgSO_4 \cdot 7H_2O$ 0.025%; KH_2PO_4 0.05%; $CaCO_3$ 0.5%, and agar 1%) and incubated at 30°C for 1 wk.

Fermentations

Enzyme production was studied in shaken-flask fermentations: 120 mL of the growth media (olive oil 1%, meat peptone 2%, NaCl 0.5%, and yeast extract 0.1%, initial pH 5.5) was inoculated with 2×10^6 spores and incubated at 30°C and 160 rpm. Fermentations were also carried out in a 5-L fermenter (BIOFLO II-New Brunswick) containing 4000 mL of an optimized growth medium at 30°C, initial pH 5.5 (not controlled). The fermenter was inoculated with 2% (v/v) of its volume with 24 h grown cells. Mixing and aeration were investigated by varying N from 50 to 300 rpm and Qa from 0.2 to 1.0 vvm.

Effect of the Carbon and Nitrogen Sources

Fermentations were carried out as in Materials and Methods except for the olive oil, which was replaced by

1. glucose 2.2%
2. glucose 1.98% plus olive oil 0.1%
3. lactose 2.2% and
4. lactose 1.98% plus olive oil 0.1%.

In all media the same C/N ratio (total carbon to total nitrogen concentration) of 9.9 was used. In a second set of experiments the effect of olive oil in the basal media was investigated by varying its concentration within the range 0.1 to 2.0%. As a consequence of keeping the peptone concentration constant and increasing the olive oil, the media C/N ratio varied from 6.2 to 13.9. To study the effect of the nitrogen source, in preliminary experiments, the meat peptone in the growth media was replaced by 2% soya and 2% casein peptones. In a second set of experiments, the concentration of soya and casein peptones were raised to 7.0 and 4.3% respectively, to equalize the media nitrogen content to the meat peptone medium *(15)*. Inorganic nitrogen was also studied, replacing meat peptone by ammonium sulfate 0.46%, ammonium nitrate 0.28%, and ammonium chloride 0.38%. The salts concentration varied to keep a C/N ratio of 9.9 in all cases.

Analytical:

Biomass Concentration

At selected time intervals, samples were withdrawn and filtered through Whatman N°44 paper. The biomass was thoroughly washed and dried at 75°C for dry weight determination.

Lipids Assay

The culture supernatant was used for gravimetric determination of lipids according to Akhtar et al. *(2)*, replacing n-hexane by a mixture of petroleum-ether and ethyl-ether (1:1).

Lipase Assay

The reaction mixture consisted of 19 mL of olive oil/arabic gum emulsion (5% olive oil and 5% arabic gum) in 100 m*M* potassium phosphate buffer, pH 7.0. This mixture was homogenized in a blender for 3 min and the enzymatic reaction started by adding 1 mL of the culture supernatant. The assay was carried out at 37°C and 200 rpm for 30 min. After this time interval, the reaction was stopped by adding 20 mL of acetone-ethanol 1:1 (v/v) and the amount of fatty acids produced was then titrated with 0.05*M* NaOH until end-point 9.5 using an automatic titration apparatus (Mettler DL21). One unit of lipase activity was defined as the amount of enzyme

that liberates 1 μmol of fatty acids equivalent per minute under the assay conditions.

Protease Assay

Proteolytic activity was determined in the culture supernatant according to Charney and Tomarelli *(16)*.

Relationship Between Cell Lysis and Protease

The relationship between cell lysis and protease concentration in the fermentation broth was evaluated in bench-scale fermenter experiments by plotting cellular decay rate $\Delta X/\Delta t$ (variation of biomass concentration since the beginning of the growth decay up to around 150 h of fermentation) against $\Delta P/\Delta t$ (variation of protease concentration in the same time interval). This determination was performed for all conditions of air supply (0.20 to 1.0 vvm) and agitation (50 to 300 rpm).

Yield Coefficient and Productivity

The overall yield coefficient (Yp/x) was defined as the maximum units of lipase produced per maximum biomass concentration. Productivity was the ratio between maximum lipase activity per fermentation time.

RESULTS AND DISCUSSION

Effect of Nitrogen Source

Penicillium restrictum was unable to grow in inorganic nitrogen, although it was reported that a strain of *P. roqueforti* was able to grow and produce lipase using ammonium sulfate as the only nitrogen source *(7)*. This inability to grow on inorganic nitrogen has also been observed for *P. roqueforti (17,18)*, *P. verrucosum* var. *cyclopium (8)*, and *P. citrinum (5)*, and could be related to transport limitation or restrictions in the metabolic pathways to incorporate inorganic nitrogen.

Table 1 shows the highest values for cell and enzyme concentrations, and normalized enzyme production (units of lipase produced per mg of cell dry weight:Yp/x), in the culture media with meat, soya, and casein peptone at variable concentrations. Lipase concentration and the Yp/x parameter were more than two times higher in meat peptone, indicating that its amino acids and/or cofactors content matches the micro-organism physiological requirements for lipase biosynthesis. These results are in accordance to the literature concerning lipase production by *Penicillium (5,7–9,11,17)* and other fungi genera *(4,10)*.

Effect of the Carbon Source

Table 1 also presents the peak data for biomass and lipase concentrations and normalized lipase production for different conditions of carbon nutrition. *Penicillium restrictum* was able to grow equally on glucose and

Table 1
Effect of the Nitrogen and Carbon Sources on Cell Growth, Overall Yield Coefficient (Yp/x) and Lipase Production by *Penicillium restrictum* in Shaken-Flask Fermentations Carried out at 30°C and 160 rpm

Nitrogen source[a]	Biomass concentration (mg/mL)	Activity (U/mL)	Yp/x (U/mg)
meat peptone (2.0%)	14.2	13.0	0.92
soya peptone (2.0%)	11.4	3.4	0.30
(7.0%)	16.5	6.6	0.40
casein (2.0%)	14.9	5.4	0.36
(4.3%)	17.5	6.2	0.35
Carbon source[b]	**Biomass concentration (mg/mL)**	**Activity (U/mL)**	**Yp/x (U/mg)**
glucose (2.2%)	14.1	1.9	0.14
olive oil (1.0%)	14.2	13.0	0.92
glucose (1.98%) + olive oil (0.1%)	13.9	1.8	0.13
lactose (2.2%)	4.3	1.7	0.40
lactose (1.98%) + olive oil (0.1%)	5.7	6.0	1.00

[a]olive oil 1% w/v was used as carbon source.
[b]meat peptone was used as nitrogen source (media with a constant ratio C/N of 9.9).

olive oil as similar biomass concentrations around 14.0 mg/mL were observed in both cases. Enzyme production however, was significantly higher (13.0 U/mL) when olive oil was used in comparison to glucose (1.9 U/mL) even when the glucose media was supplemented with 0.1% olive oil. Accordingly, lipase production by *P. restrictum* seems to be glucose regulated. Similar results were observed for *Penicillium citrinum* when glucose or sucrose were used even in the presence of triglycerides (*5*). These

findings indicate that the avoidance of a repressive carbon source is of paramount relevance for lipase production.

The use of lactose resulted in poor cell growth (4.3 mg cell/mL) probably because of the inability of the micro-organism to metabolize lactose. Cell growth was, therefore, limited to the peptone availability. Concerning lipase production, although only basal levels were observed (1.7 U/mL), the normalized production (0.4 U/mg cell) reflected the absence of glucose repression. When lactose was supplemented with olive oil at 0.1%, an improvement in cell growth (5.7 mg/mL), enzyme production (6.0 U/mL), and normalized production (1.0 U/mg) were observed. This beneficial effect can be explained by the higher carbon availability to the cell metabolism or oil induction of enzyme biosynthesis *(3,6,19)*. In further experiments, similar enzyme levels were observed using peptone 2% w/v and olive oil 0.1% w/v (Fig. 1). In conclusion, olive oil showed to be more appropriate for both cell growth and lipase production, glucose repressed the enzyme synthesis, and lactose was poorly metabolized. Although the possible causes to these physiological responses were discussed, it is important to point out that the measurement of enzyme concentration in culture supernatant may not be directly related to its biosynthesis, thus, considerations related to regulation of gene expression are open to further discussions.

Effect of Olive Oil Concentration on Lipase Production and Cell Growth

Figure 1 compares the highest data for biomass concentrations and lipase accumulation for the range of oil concentrations studied. There is a clear positive effect of the concentration increase on cell growth indicating that the basal medium (1% olive oil, 2% peptone) was carbon limited. In response to the increase in oil availability from 0.1 to 2.0% (media C/N ratio from 6.2 to 13.9), biomass varied steadily from 5.5 to 22.4 mg/mL. The overall positive effect of oil concentration on cell growth was not likewise observed on lipase production as peak concentration of 13.0 U/mL were obtained with 0.5 and 1.0% olive oil decreasing afterwards. In conclusion, a threshold olive oil availability, related to the medium C/N ratio, would trigger a metabolic shift in *P. restrictum* towards cell growth in detriment of enzyme production. Lower enzyme activity at higher oil concentrations (C/N > 9.9) are explained by some authors as enzyme inhibition by free fatty acids. This effect, however, has not been properly characterized *(2,20)*.

Lipase and Protease Production in a Bench-Scale Fermenter

A typical fermentation time course for lipase production by *P. restrictum* is shown in Fig. 2. Using optimized inoculum and medium conditions, the experiments were carried out at 200 rpm and 0.5 vvm. The

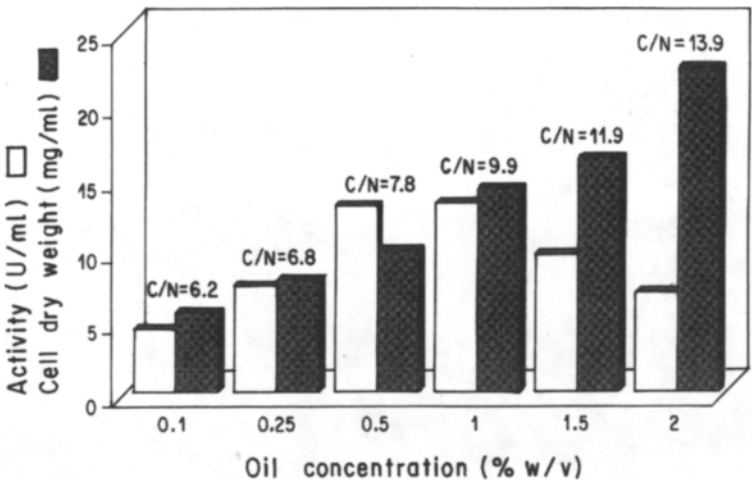

Fig. 1. Effect of olive oil concentration (w/v) on biomass and lipase accumulation in *Penicillium restrictum* shaken-flask fermentations (30°C and 160 rpm), having meat peptone 2% w/v as nitrogen source. Media carbon to nitrogen ratios (C/N) are presented for each case.

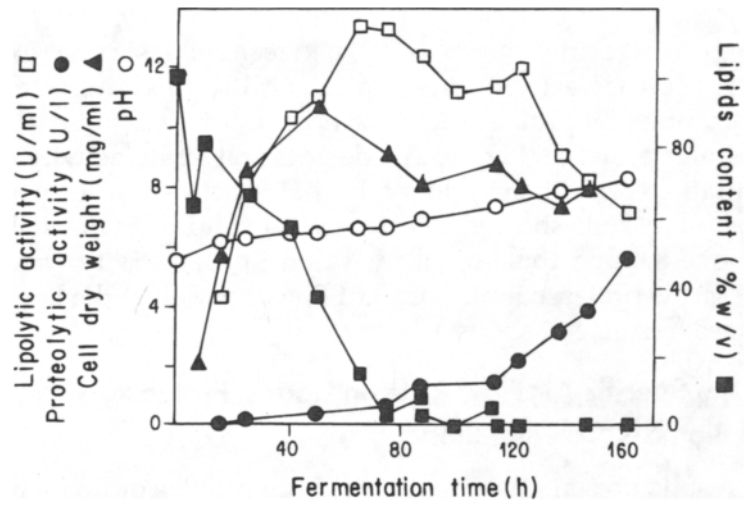

Fig. 2. Typical time course for cell growth, lipase production, protease accumulation, and lipids consumption by *Penicillium restrictum* in a 5-L bench-scale fermenter. Experiment carried out at 200 rpm, 0.5 vvm, 30°C, and initial pH 5.5.

maximum lipolytic activity (13.4 U/mL) was observed after 70 h of cultivation, which was coincident with the depletion of the carbon source (lipids). Lipase production showed to be growth associated, although a small increase on enzyme activity was observed after the end of the cell-growth phase. This increase could be related to the intracellular lipase release upon cell lysis that was observed after 40 h of fermentation. It was

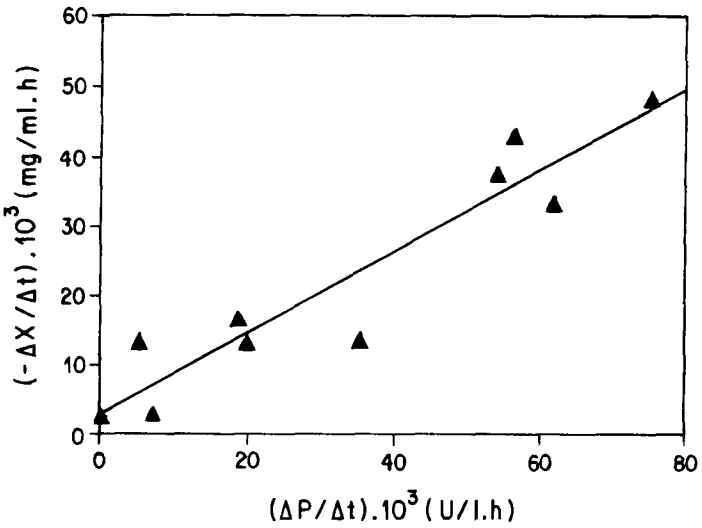

Fig. 3. Relationship between cellular decay rate ($-\Delta X/\Delta t$) and protease production rate ($\Delta P/\Delta t$) in *Penicillium restrictum* fermentations using a 5-L bench-scale fermenter. Working conditions according to text.

observed a direct relationship ($r = 0.92$) between cell lysis rate ($\Delta x/\Delta t$) and the rate of protease activity increase ($\Delta P/\Delta t$) at the later fermentation stage, indicating that protease activity increase was a result of the release of intracellular protease (Fig. 3). The decrease on lipase activity after 70 h of fermentation could be explained by pH inactivation, proteolysis, or both. Previous results showed that *P. restrictum* lipase is not stable at pH values above 8.0 and that the decrease on lipase activity was reduced when the serine protease inhibitor (PMSF) was added at the later fermentation stages (results not shown).

Effect of the Specific Air Flow Rate on Lipase, Protease, and Biomass Accumulation

The results presented in Table 2 and the profiles of lipase activity in experiments carried out using different specific air flow rates at 200 rpm presented in Fig. 4A, indicate that Qa variation had no significant effect on lipase production. However, Qa increase had a positive effect on cell growth and protease accumulation (Table 2 and Fig. 4B). A higher oxygen availability improved biomass accumulation and consequently the amount of protease released upon cell lysis. Isobe et al. *(21)*, working with *P cyclopium*, utilized a high Qa value (1.0 vvm) to increase cell growth for the inoculum preparation and then a reduced Qa (0.25 vvm) during the fermentation. Such procedure is in accordance to our results since a higher oxygen availability promoted a metabolic shift toward cell growth in detriment of lipase production.

Table 2
Effect of the Impeller Speed and the Specific Air Flow Rate on Lipolytic Activity, Biomass Concentration, Overall Yield Coefficient (Yp/x), and Productivity[a]

N (rpm)	Qa (vvm)	Maximum lipolytic activity (U/mL)	Maximum biomass concentration (mg/mL)	Yp/x (U/mg)	Productivity (U/L.h)
50	0.20	3.5	5.6	0.63	30
50	0.50	4.9	5.9	0.83	42
100	0.20	8.9	6.0	1.48	75
100	0.50	9.3	8.5	1.09	78
100	1.00	9.1	11.5	0.79	82
200	0.20	12.3	7.2	1.71	141
200	0.50	13.4	9.3	1.44	209
200	0.75	13.3	11.7	1.14	147
200	1.00	11.4	13.5	0.84	208
300	0.20	11.3	13.0	0.87	246
300	0.50	12.2	13.9	0.87	306
300	1.00	13.0	15.1	0.86	263

[a]Maximum data for enzyme and biomass concentration in fermentations carried out in a 5-L bench-scale fermenter at 30°C and initial pH 5.5.

Effect of Agitation Speed on Lipase, Protease, and Biomass Accumulation

Figure 5A presents the effect of N on lipase production using 0.5 vvm. A positive effect of agitation on lipase production was observed up to 200 rpm. At this impeller speed, maximum enzyme activity was improved 1.4 and 2.7 times in comparison to the lipase concentration with 100 and 50 rpm, respectively. An acceleration was also observed on lipase accumulation and/or on its release to the medium as an anticipation of the activity peak was observed when N was increased from 50 to 300 rpm. A similar

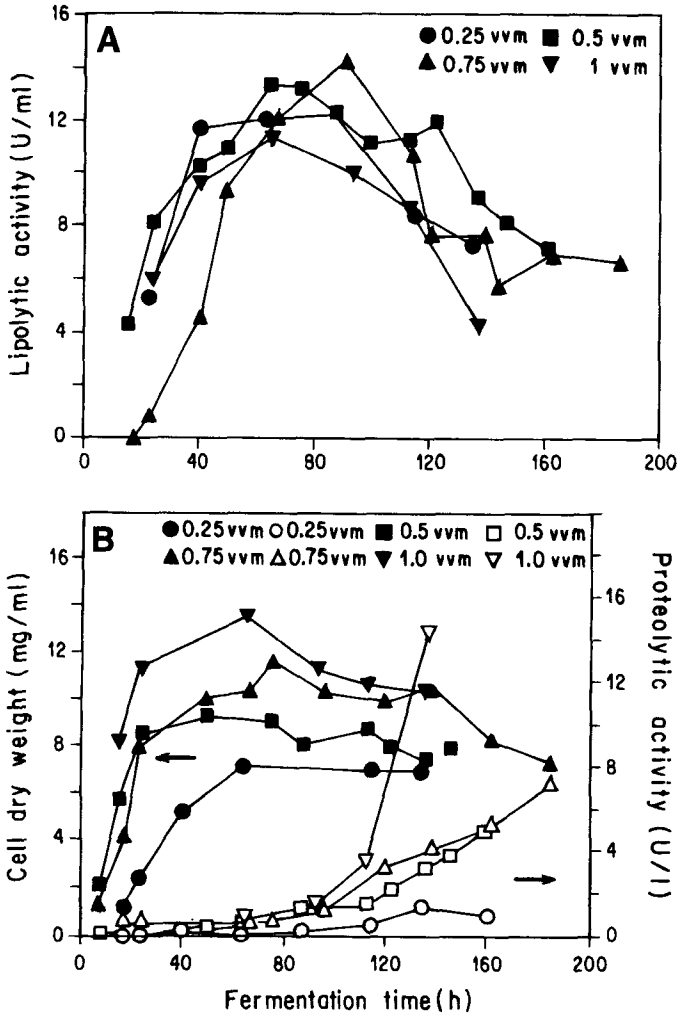

Fig. 4. Effect of the specific air flow rate on lipase production **(A)**, cell growth and protease accumulation **(B)** by *Penicillium restrictum*. Experiments carried out in a 5-L instrumented fermenter (200 rpm, 30°C, and initial pH 5.5).

trend was observed for *Geotrichum candidum* cultivation in shaken flasks (*22*). This effect seems to be related to the improvement of the mass transfer conditions in the fermenter. As lipids are water nonmiscible carbon sources, their availability for microbial consumption is favored by the turbulence attained in the medium. At higher impeller speeds, the mixing improvement favors substrate availability being beneficial to a higher and faster cell growth and lipase production. It is well-established that N is a relevant operational parameter, mainly in highly viscous nonNewtonian fluids, as is the case of filamentous fungi fermentation broths. Figure 5B shows that protease activity increased upon increments on the impeller speed, which can be related to both higher biomass accumulation and cell lysis because of mycelium fragmentation.

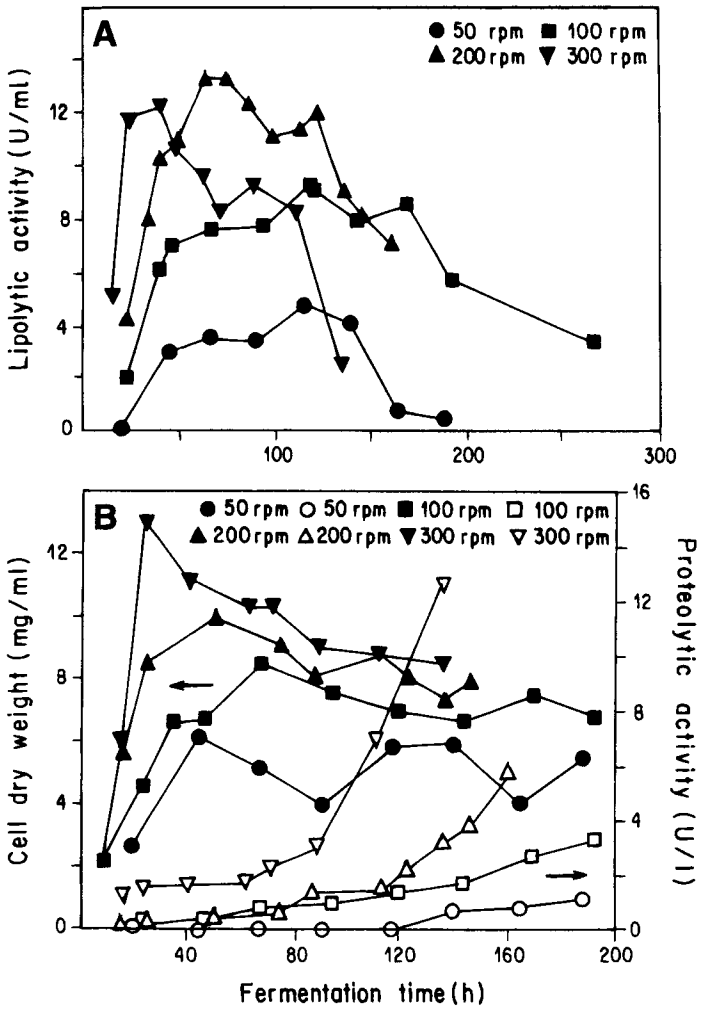

Fig. 5. Effect of the impeller speed on lipase production **(A)**, cell growth and protease accumulation **(B)** by *Penicillium restrictum*. Experiments carried out in a 5-L instrumented fermenter (0.5 vvm, 30°C, and initial pH 5.5).

Effect of Air Supply and Agitation on the Yield Coefficient and Productivity

According to data presented in Table 2, productivity was not significantly affected by Qa. Figure 4A that compares enzyme concentration profiles for 200 rpm at variable Qa values shows that similar peak enzyme concentrations were observed in all cases at around 75 h of fermentation. Agitation showed a marked effect on productivity as by the use of 50, 100, 200, and 300 rpm, the average values obtained were of 36, 78, 176, and 272 U/L.h., respectively. This was a result of the anticipation of the activity peak from 120 h (50 rpm) to 30 h (300 rpm) and to the increase of the maximum activity value by a factor of 2.5 (Fig. 5A). The yield coefficient

showed a decreasing trend with increase in the air flow rate, as observed in the experiments conducted at 100 and 200 rpm. For these two conditions the maximum lipolytic activity was not affected by Qa, although, maximum biomass concentration raised markedly. These results indicate that fungal growth was stimulated by a higher oxygen supply, and that lipase production was favored by oxygen limitation. At impeller speed of 300 rpm both cell growth and lipase production were not affected by air flow rate, indicating that a saturation condition was attained in terms of oxygen and nutrients availability.

A positive effect on the yield coefficient was observed with the increase on the impeller speed up to 200 rpm. In the range of 50 to 200 rpm (0.5 vvm), the maximum lipolytic activity increased by a factor of about 2.7, whereas the maximum biomass concentration increased 1.6 times. Consequently, the yield coefficient increased about 1.7 times. As previously commented, this result seems to be related to a better availability of nutrients caused by improved mixing conditions. Beyond 300 rpm no improvement on lipase production was observed and consequently the yield coefficient decreased.

In conclusion, the results hereby presented indicated that the best results concerning lipase production were achieved in fermentations conducted at 30°C when meat peptone (2% w/v) and olive oil (1% w/v) were used as nitrogen and carbon sources (medium with a C/N ratio of 9.9), initial pH 5.5 natural pH fermentations. Using these conditions, the optimized agitation and aeration in the bench-scale fermenter were 200 rpm and 0.5 vvm.

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