Control of Lipase Production by *Rhizopus oligosporus* under Various Growth Conditions

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Some factors influencing the growth and production of extracellular lipase by *Rhizopus oligosporus* were studied. Highest yields of enzyme were obtained when Tweens were the carbon source. Soybean meal extract supported good growth and enzyme production. Carbohydrates, vegetable oils, proteins or amino acids did not stimulate lipase production. The fungus grew well with carbohydrate- or protein-supplemented media but not with oils, unless emulsified with a non-metabolizable gum. The production of biomass in static cultures was maximum at 35–40 °C after 4 d at pH 5.5. The yield of lipase was maximum at 25 °C after 3 d at pH 6.5. Shaking cultures enhanced growth but decreased lipase production.

INTRODUCTION

Rhizopus oligosporus is a fungus used to produce tempeh, a fermented soybean food product (Nahas & Machado, 1982). This fermentation is reported to enhance the digestibility of soybean (Wang & Hesseltine, 1965), and the lipolytic activity of the fungus (Wagenknecht *et al.*, 1961) contributes to imparting desirable flavours. Although a considerable amount of information is available on the fermentation of soybean by *R. oligosporus*, few studies have been done in chemically defined media.

Factors affecting microbial extracellular lipase production have been widely studied in bacteria (Lawrence *et al.*, 1967; Mates & Sudakevitz, 1973), moulds (Chander *et al.*, 1980; Chopra & Chander, 1983) and yeasts (Ota *et al.*, 1968*a, b*). Lipases from two *Rhizopus* spp. have been purified (Iwai & Tsujisaka, 1974; Aisaka & Terada, 1981). Few studies have been made with *R. oligosporus* to examine the control of lipase production (Smith & Alford, 1968) and the growth of the fungus (Sorenson & Hesseltine, 1966). This study was therefore initiated to investigate the effect of different growth media and conditions on lipase production by *R. oligosporus*.

METHODS

Chemicals. Olive oil was purchased from Sigma. All other chemicals were of analytical reagent grade.

Culture and inoculum. The organism used was *Rhizopus oligosporus* Saito, NRRL 2710. Stock cultures were maintained by weekly transfers on Sabouraud agar slants. Inoculum was prepared by washing two freshly subcultured slants with sterile distilled water and filtering through a double layer of commercial gauze to remove contaminating mycelia. Spore suspensions were adjusted to 2×10^7 ml⁻¹.

Basal medium. Citrate mineral salts medium (adjusted to pH 6.0) was prepared as described by Crocken & Nyc (1963). When the effect of the carbon source was being considered, carbohydrates, lipids, proteins or amino acids were added. The carbohydrates were sterilized separately and added to the sterile medium before inoculation. Defatted soybean meal extract was prepared by the procedure of Iwai & Tsujisaka (1974). When the effect of nitrogen source was being studied, ammonium nitrate was replaced with the nitrogen compounds and 1% (w/v) glucose was used as the carbon source.

Culture method. A spore suspension (0.5 ml) was inoculated into each Petri dish (14 cm diameter) containing 50 ml medium supplemented with the carbon and/or nitrogen sources. After 72 h, the cultures were filtered through a Büchner funnel, and the mycelial pads were washed with water and pressed between several sheets of

filter paper. Mycelial mass was expressed as dry weight of the pressed mycelial pads after heating for 24 h at 98 °C.

Lipase assay. Lipase activity was determined as described by Akhtar *et al.* (1980), with some modifications. The substrate was a 5% (v/v) olive oil emulsion prepared in 1% (w/v) gum acacia by homogenizing in a top-drive homogenizer for 6 min; the pH was adjusted to 8.5. The reaction mixture contained 2.5 ml substrate, 3.5 ml of 50 mM-Tris/HCl buffer, pH 8.5, and 2.0 ml of enzyme solution, diluted twofold after the pH value had been adjusted to 8.5. After incubation at 37 °C for 3 h, the reaction was terminated by the addition of 10 ml acetone/ethanol (1:1, v/v). The resulting mixture was then titrated to pH 10.5 against 0.05 M-NaOH. Blanks obtained with enzyme samples boiled for 4 min were subtracted, and the activities were expressed as μmol free fatty acids released. Determinations were done in duplicate. One unit (U) of lipase activity was defined as the amount of enzyme releasing 1 μmol free fatty acids h^{-1} . Specific activities were expressed as $\text{U (mg dry wt mycelium)}^{-1}$.

RESULTS

A range of different carbon sources were tested for their ability to support growth and enzyme production by *R. oligosporus*. Good growth but poor enzyme production was obtained on media supplemented with carbohydrates (Table 1). Mycelial growth and enzyme production on lipid-supplemented media were both very poor except for the polyoxyethylenesorbitans, which gave the highest enzyme activities observed: about 62 U mg^{-1} with Tween 20 and 67 U mg^{-1} with Tween 80 (Table 2). Acetic and palmitic fatty acids did not support any mycelial growth. The low mycelial biomass obtained in media supplemented with oils was due to limited availability

Table 1. *Effect of different carbohydrates in the medium on the growth and production of lipase by R. oligosporus*

The organism was grown at 30 °C for 72 h in 50 ml of medium; the results are means $\pm$ SEM of four separate cultures. Carbon sources were at 1% (w/v). This organism was also tested on lactose, sucrose, and glycerol but did not grow.

Carbon source	Dry wt. (mg ml^{-1})	Final pH of medium	Lipase activity [U (ml medium) $^{-1}$]	Lipase specific activity [U (mg mycelium) $^{-1}$]
Xylose	1.5 $\pm$ 0.028	5.3	4.7 $\pm$ 0.14	3.1
Glucose	2.0 $\pm$ 0.007	4.2	3.8 $\pm$ 0.16	1.9
Galactose	1.1 $\pm$ 0.019	5.6	5.0 $\pm$ 0.24	4.4
Fructose	1.9 $\pm$ 0.021	4.7	1.0 $\pm$ 0.14	0.5
Maltose	1.6 $\pm$ 0.049	5.0	2.7 $\pm$ 0.14	1.7
Starch	1.8 $\pm$ 0.047	4.9	2.3 $\pm$ 0.14	1.3
Mannitol	1.6 $\pm$ 0.006	5.4	3.6 $\pm$ 0.16	2.3

Table 2. *Effect of different lipid sources in the medium on the growth and production of lipase by R. oligosporus*

Assay conditions were as in Table 1. Carbon sources were at 0.5% (v/v). Oil emulsions were prepared in 0.7% (w/v) gum acacia.

Carbon source	Dry wt. (mg ml^{-1})	Final pH of medium	Lipase activity [U (ml medium) $^{-1}$]	Lipase specific activity [U (mg mycelium) $^{-1}$]
Cotton oil	0.2 $\pm$ 0.007	5.8	1.2 $\pm$ 0.10	7.3
Emulsified cotton oil	1.2 $\pm$ 0.006	5.5	15.3 $\pm$ 0.21	13.2
Rice oil	0.3 $\pm$ 0.008	5.7	4.5 $\pm$ 0.17	13.9
Emulsified rice oil	0.8 $\pm$ 0.005	5.6	18.0 $\pm$ 0.19	21.4
Maize oil	0.2 $\pm$ 0.005	5.8	2.2 $\pm$ 0.10	12.4
Emulsified maize oil	0.9 $\pm$ 0.001	5.6	16.1 $\pm$ 0.34	17.4
Olive oil	0.4 $\pm$ 0.003	5.6	4.2 $\pm$ 0.22	9.5
Emulsified olive oil	1.1 $\pm$ 0.030	5.6	16.4 $\pm$ 0.21	14.8
Soybean oil	0.4 $\pm$ 0.001	5.6	3.6 $\pm$ 0.21	9.1
Emulsified soybean oil	1.1 $\pm$ 0.039	5.7	10.6 $\pm$ 0.16	9.7
Tween 20	0.4 $\pm$ 0.018	5.6	27.0 $\pm$ 0.19	61.7
Tween 80	0.8 $\pm$ 0.018	5.7	55.2 $\pm$ 0.33	66.7

Fig. 1. Production of lipase in cultures of *R. oligosporus* grown on soybean meal extract (30%, v/v) plus glucose (0.5–2.0%, w/v) at 30 °C for 72 h. ●, Lipase activity; ○, dry weight of mycelium; ▲, final pH.

Table 3. *Effect of various organic nitrogen compounds in the medium on the growth and production of lipase by R. oligosporus*

Assay conditions were as in Table 1. Carbon sources were at 1% (w/v), except for yeast extract, peptone and tryptone that were at 2% (w/v), and soybean meal extract at 30% (v/v).

Carbon source	Dry wt. (mg ml ⁻¹)	Final pH of medium	Lipase activity [U (ml medium) ⁻¹]	Lipase specific activity [U (mg mycelium) ⁻¹]
Glutamic acid	0.3 ± 0.008	8.0	5.6 ± 0.63	17.8
Aspartic acid	0.3 ± 0.010	8.7	4.1 ± 0.39	14.6
Arginine	0.3 ± 0.015	6.4	6.6 ± 0.52	20.7
Casein	1.5 ± 0.039	8.1	17.3 ± 0.59	11.3
Casein hydrolysate	2.0 ± 0.012	8.0	29.5 ± 0.38	14.9
Soybean meal extract	0.7 ± 0.019	7.1	30.8 ± 0.46	47.3
Yeast extract	2.2 ± 0.019	8.3	6.3 ± 0.76	3.0
Gelatine	0.5 ± 0.021	7.6	11.9 ± 0.63	24.9
Peptone	1.2 ± 0.020	8.2	19.3 ± 0.69	16.1
Tryptone	2.1 ± 0.064	8.2	23.6 ± 0.98	11.2

of these carbon sources to the fungus. When emulsified with gum acacia, oils supported good growth and enzyme production (Table 2). Gum acacia on its own did not support growth. The final pH values of the growth media did not change significantly in the presence of lipids (Table 2); however, carbohydrate-supplemented media decreased the pH to values ranging between 4.2 and 5.4. *R. oligosporus* was also grown in media containing soybean meal extract in combination with various concentrations of glucose as carbon sources. The results showed that mycelial growth was stimulated by about fivefold when 2% (w/v) glucose was added to the medium (Fig. 1). However, lipase production decreased by about ninefold on 2% glucose as compared with 30.8 U ml⁻¹ in its absence.

Data concerning the effect of amino acids and proteins as carbon sources on the growth of the mould and lipase production are presented in Table 3. Urea was tested but failed to support any growth. Low levels of biomass and enzyme activity were obtained with the amino acids. All the proteins tested, with the exception of soybean meal extract and gelatin, stimulated fungal growth. Significant biomass production was observed with casein hydrolysate, yeast extract and tryptone (Table 3). The soybean meal extract promoted maximum lipase production per unit of growth with *R. oligosporus* (about 47 U mg⁻¹) (Table 3). The production of lipase in media supplemented with amino acids or proteins ranged between 3 and 21 U mg⁻¹.

Fig. 2. Effect of period of incubation on the production of lipase by *R. oligosporus* grown on soybean meal extract (30%, v/v), at 30 °C. ●, Lipase activity; ○, dry weight of mycelium; ▲, final pH.

Fig. 3. Effect of temperature of incubation on the production of lipase by *R. oligosporus* grown on soybean meal extract (30%, v/v) for 72 h. ●, Lipase activity; ○, dry weight of mycelium; ▲, final pH.

Salts, amino acids, proteins and urea were used as nitrogen sources in media supplemented with 1% glucose (Table 4). With tryptone as nitrogen source, *R. oligosporus* showed excellent growth, with the biomass production enhanced 182% as compared to the production with ammonium nitrate. However, the lipase activity per mg dry weight was poor (Table 4). Proteins both as carbon or nitrogen sources raised the pH values of the culture media, while the amino acids tested increased the pH values only as carbon sources (Tables 3 and 4).

The effect of the initial pH on the growth of mould and lipase secretion in soybean meal extract medium was tested between pH 4 and 7. Although the optimum pH for growth was at 5.5, enzyme production was highest at pH 6.5.

Lipase production was tested over a 9 d period by using soybean meal extract in the culture medium (Fig. 2). Lipolytic activity in the cell-free culture filtrates was detectable very early in the incubation period. The maximum amount of lipase activity was obtained after 3 d with recoverable enzyme activity gradually decreasing thereafter and reaching about 19% of the maximum after 9 d. Biomass production increased up to the fourth day, after which the culture reached the stationary phase (Fig. 2). The decrease in enzyme activity occurred simultaneously with the onset of the stationary phase of fungal growth.

The optimum temperature for lipase production by the fungus was 25 °C (Fig. 3); however, the lipolytic activity at 20 and 30 °C was about 85 and 75%, respectively, of the optimum. At

Table 4. *Effect of different nitrogen sources on the medium on the growth and production of lipase by R. oligosporus*

Assay conditions were as in Table 1. Salts, amino acids and urea concentrations were 12.5 mM. Casein, casein hydrolysate, peptone and tryptone were at 2% (w/v). Soybean meal extract was at 30% (v/v). Glucose (1%, w/v) was the carbon source.

Nitrogen source	Dry wt. (mg ml ⁻¹)	Final pH of medium	Lipase activity [U (ml medium) ⁻¹]	Lipase specific activity [U (mg mycelium) ⁻¹]
Ammonium nitrate	2.0 ± 0.009	4.2	3.8 ± 0.16	1.9
Ammonium chloride	1.8 ± 0.021	4.8	—	—
Ammonium sulphate	3.0 ± 0.018	6.0	7.3 ± 0.29	2.4
Urea	2.6 ± 0.048	5.8	4.9 ± 0.25	1.9
Arginine	3.0 ± 0.046	7.2	11.8 ± 0.16	3.9
Glycine	1.8 ± 0.001	5.6	2.7 ± 0.33	1.5
Aspartic acid	1.9 ± 0.016	6.1	2.0 ± 0.14	1.2
Casein	5.0 ± 0.002	7.9	11.9 ± 0.39	2.4
Casein hydrolysate	4.9 ± 0.075	7.7	48.8 ± 0.93	9.9
Soybean meal extract	3.3 ± 0.014	6.0	34.0 ± 0.24	10.5
Peptone	4.0 ± 0.050	7.7	16.1 ± 0.16	4.0
Tryptone	5.5 ± 0.051	7.5	40.5 ± 0.29	7.4

Table 5. *Effect of aeration on growth and lipase production by R. oligosporus*

Assay conditions were as in Table 1. Carbon sources were at 0.5% (v/v) except for soybean meal extract that was at 30% (v/v). One set was incubated without shaking and the second aerated by reciprocal shaker (110 cycles min⁻¹).

	Dry wt. (mg ml ⁻¹)	Final pH of medium	Lipase activity [U (ml medium) ⁻¹]	Lipase specific activity [U (mg mycelium) ⁻¹]
Soybean oil				
Stationary	0.5 ± 0.016	5.6	4.6 ± 0.21	10.2
Shaker	1.9 ± 0.008	5.2	15.9 ± 0.25	8.2
Soybean meal extract				
Stationary	0.6 ± 0.010	7.1	31.0 ± 0.24	51.2
Shaker	1.0 ± 0.006	7.2	14.9 ± 0.27	15.1
Tween 80				
Stationary	0.8 ± 0.008	5.7	55.1 ± 0.43	71.7
Shaker	0.9 ± 0.042	5.6	47.5 ± 0.55	49.0

temperatures above 30 °C, the recoverable lipolytic activity was very low (Fig. 3). Increasing temperatures between 20 and 40 °C also increased mycelial growth at the time of sampling and consequently increased the pH of the culture medium.

To test the effect of aeration, cultures were incubated at 30 °C for 3 d in a reciprocal shaker set at 110 cycles min⁻¹. It becomes evident from these results that aeration influences mycelial growth. Thus, in soybean oil-supplemented medium, fungal growth increased about fourfold, whereas with the other sources tested, the increment was about one to twofold. This greater growth resulted in higher enzyme production (U ml⁻¹); however, when lipolytic activity was expressed as U (mg dry wt)⁻¹, a significant reduction was observed (Table 5).

DISCUSSION

The present results show that mycelium (dry wt) and lipase production vary widely under various growth conditions. Thus, carbohydrates were good carbon sources for growth of *R. oligosporus* but low lipase production was obtained. These results agree with those of Alford & Pierce (1963), Ota *et al.* (1968*a*) and Tsujisaka *et al.* (1973) for lipase in bacteria, yeast and fungi, respectively.

To further understand the effect of carbohydrates, the growth medium was supplemented with soybean meal extract and increasing concentrations of glucose. Lipase production was strongly repressed by higher concentrations of glucose (Fig. 1). Peters & Nelson (1948) demonstrated that the presence of glucose in amounts exceeding 0.05% is detrimental to lipase production by *Mycotorula lipolytica*. Lipase production in oil-supplemented medium was much lower than in media containing Tween 80. This decrease of lipase production is comparable with that reported by Chander *et al.* (1980), who concluded that butter oil and olive oil inhibited lipase activity by 53 and 63%, respectively. Similar observations were made by Lawrence *et al.* (1967) and Eitenmiller *et al.* (1970). However, lipase activity in oil-supplemented medium is influenced by exposure of the lipid substrate to attack by the fungus so that increased dispersion of the substrate enhanced growth and enzyme production. Unlike the other lipids used, the Tweens significantly enhanced lipase production. These results are in accordance with those of Ota *et al.* (1968*a, b*), who found that Tweens are able to induce lipase production.

In medium containing proteins either as carbon source or as nitrogen source, lipase secretion was not stimulated, except with soybean meal extract, which resulted in high levels of lipase production (Table 3). These results were similar to those observed by Chopra & Chander (1983) who found about twofold enhanced lipase production with cornsteep liquor over the other proteins tested. Thus, lipase production with soybean meal extract is comparable with that in the presence of Tweens. *R. oligosporus* is used for soybean fermentation not only because of its high proteolytic and lipolytic activity (Wagenknecht *et al.*, 1961; Wang & Hesseltine, 1965) but also for improvement of the final flavour (Nahas & Machado, 1982). The results concerning lipolytic activity in more defined media are in agreement with findings obtained with solid substrates.

Lipase production is also influenced by other factors. Thus, similarly to other fungi (Stern *et al.*, 1954; Chander *et al.*, 1980) the optimum pH values for growth and lipase production were 5.5 and 6.5, respectively. High incubation temperatures (35–40 °C) enhanced the growth of *R. oligosporus* but enzyme production was better at lower temperatures (25 °C). High temperatures may possibly lead to a denaturation of the enzyme. These results are similar to those obtained with *Penicillium roqueforti* (Eitenmiller *et al.*, 1970) but differ from those obtained for *Mucor mucedo* by Stern *et al.* (1954), who reported inhibition of enzyme activity at temperatures below 30 °C. Growth of *R. oligosporus* in soybean oil culture medium was significantly enhanced in shaking cultures, confirming results obtained by Sorenson & Hesseltine (1966). Thus, in addition to the aeration effect, shaking may have created conditions of higher availability of the carbon source to the fungus. The lipolytic activity (U ml⁻¹) was also higher than in static cultures. However, enzyme production per unit weight was significantly reduced. This may have been due to a possible denaturing effect of shaking (Vadehra & Harmon, 1969). These results confirm those reported by Chopra & Chander (1983) for *Syncephalastrum racemosum*, which produced more lipase when the culture was static.

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