World Journal of Microbiology & Biotechnology 2005 **21**: 33–38
DOI: 10.1007/s11274-004-1556-2

Preparation and testing of *Sardinella* protein hydrolysates as nitrogen source for extracellular lipase production by *Rhizopus oryzae*

Sofiane Ghorbel[1], Nabil Souissi[1], Yosra Triki-Ellouz[1], Laurent Dufossé[2], Fabienne Guérard[2] and Moncef Nasri[1,*]
[1]*Laboratoire de Génie Enzymatique et de Microbiologie – Ecole Nationale d'Ingénieurs de Sfax. B.P. "W" 3038 Sfax, Tunisie*
[2]*Laboratoire de Microbiologie Appliquée, LUMAQ, E.A. 2651, I.U.P. Innovation en Industries Alimentaires, Creac'h Gwen, F-29000 Quimper, France*
**Author for correspondence: Tel.: +216-74-274-088, Fax: +216-74-275-595, E-mail: moncef.nasri@enis.rnu.tn*

Received 5 February 2004; accepted 19 May 2004

Keywords: Fish, lipase, peptone, protein hydrolysate, *Rhizopus oryzae*, Sardinelle

Summary

Various fish protein hydrolysates (FPH) from sardinella (*Sardinella aurita*) were used as nitrogen sources for the production of extracellular lipase by the filamentous fungus *Rhizopus oryzae*. The best results were obtained with defatted meat–fish protein hydrolysates (DMFPH), indicating the presence in the lipid fraction of some constituents which may repress lipase synthesis. Furthermore, it was found that the extensive hydrolysis of fish proteins resulted in a higher lipase production. The use of 40 g DMFPH l^{-1} for the growth of *Rhizopus oryzae* in medium R1 resulted in a lipase production of 394 U ml^{-1}, higher than the yield obtained with standard soy peptone as nitrogen source (373 U ml^{-1}). The most appropriate medium for the growth and the production of lipase is composed only of 24 g DMFPH l^{-1} and 10 g glucose l^{-1}, indicating that the strain can obtain its nitrogen and salts requirements directly from fish substrate.

Introduction

The nitrogen source is usually the most expensive component of bacterial growth media. Organic nitrogen substrates, such as peptones, are widely used in many biological and biotechnological applications, such as microbial biomass production (Singh *et al.* 1995), and metabolite biosynthesis including enzymes (Haltrich *et al.* 1994; Rapp 1995). At present, peptones are obtained from casein, soy protein, gelatin and meat (Reed Hamer and West 1994; Reissbrodt *et al.* 1995).

Lipases are one of the most important groups of industrial enzymes and they are widely used for biotechnological applications in the food and dairy industries (Reed 1975; Gandhi 1997), leather processing (Iwai & Tsujusaka 1984), detergents (Saad 1995) and pharmaceuticals.

Many strains are used for the industrial production of lipases such as *Rhizopus delemar*, *Aspergillus niger*, *Geotrichum candidum*, *Candida rugosa* and *Chromobacterium viscosum* (Gandhi 1997). *Rhizopus oryzae* produces two lipases, i.e. intra- and extra-cellular and their production was affected by the composition of the medium. Among the several substrates tested glycerol was the only stimulator of extracellular enzyme production (Salleh *et al.* 1993). Intracellular lipase production by *Rhizopus* species was strongly enhanced when cells were immobilized as compared to free cells (Nakashima *et al.* 1990). The highest production of lipolytic activity, with *Rhizopus oryzae*, was achieved on rich peptone media (Ben Salah *et al.* 1994).

The cost of lipase production may be reduced considerably by the use of inexpensive raw materials, promoting new industrial applications and making enzymatic processes more competitive. Due to its good amino acid balance and high protein content, fish materials represent a potential source of industrial peptones. Fish protein hydrolysates (FPH) have only been used to a minor extent, such as animal feeding (Kristinsson & Rasco 2000). The use of fish protein hydrolysates for maintaining the growth of different microorganisms has received a great of attention (Clausen *et al.* 1985; Gildberg *et al.* 1989; De la Broise *et al.* 1998; Dufossé *et al.* 2001), but only limited number of reports have been published about the application of this substrate to metabolite production (Coello *et al.* 2000).

Peptones from fish species have recently appeared in media catalogues (Dufossé *et al.* 1997). Thus, it would be very advantageous if low value fish materials (fish species and fish processing by-products), could be converted into more valuable products, such as peptones for fermentation media (Rodriguez-Estrada *et al.* 1994).

In this study we report the biosynthesis of lipase by *Rhizopus oryzae* grown on *Sardinella* protein hydrolysates.

Materials and methods

Microorganism

Rhizopus oryzae was provided by the Laboratoire de Biochimie et de Lipolyse Enzymatique – Ecole Nationale d'Ingénieurs de Sfax, TUNISIE.

Materials

Alcalase 2.4L and Neutrase were provided by Novo Nordisk (Denmark). MN7 protease was produced by *Pseudomonas aeruginosa* MN7, an alkaliphilic strain isolated from tannery wastewater. J21 enzyme was produced by *Bacillus* sp. isolated from Sfax fishing port. Sardinella (*Sardinella aurita)* was caught in Mediterranean Sea.

Cultivation and media

The initial medium (R1) used for lipase production by *R. oryzae* was composed of (g l^{-1}): soy peptone, 40; glucose, 10; NaCl, 3; K_2HPO_4, 2; $MgSO_4{\cdot}7H_2O$, 0.5; $CaCl_2{\cdot}2H_2O$, 0.1 and sodium citrate 1.5 (Ben Salah *et al.* 1994). Media were autoclaved at 120 °C for 20 min. The fish medium contained 40 g l^{-1} of fish substrate as nitrogen source.

Cultivations were performed in a rotatory shaker (150 rev min^{-1}) for 72 h at 30 °C, in 500 ml Erlenmeyer flasks with 100 ml of medium. The cultures were centrifuged to remove fungal cells, and the supernatants were used for estimation of lipolytic activity. All experiments were carried out in duplicate and repeated at least twice.

Preparation of fish powders

To obtain fish–meat powders, heads and viscera were first eliminated. The raw material was then heated till boiling. The bones were removed and the cooked meat was pressed to remove water and fat. The resulting pressed product was minced in a meat grinder, then dried at 80 °C for 24–48 h. The dried fish preparation was minced again to obtain a fine powder, then stored in glass bottles at room temperature. In order to obtain whole fish powder, raw material was cooked, pressed, minced and then dried.

Preparation of defatted fish protein hydrolysate (DFPH)

Fish powders, obtained as described above, were first defatted by extraction with acetone and then subjected to hydrolysis with Alcalase. Hydrolysis of fish proteins was carried out in a pH-stat at pH 8.0 and 60 °C. Defatted fish substrate was suspended in water at a concentration of 8% (w/v). The pH was adjusted to 8.0 by 1 M NaOH, then the enzyme was added. The digestion mixture was incubated for about 3 h. Once the hydrolysis had been completed (the hydrolysis degree remained constant) the mixture was heated in a boiling water bath for 5 min to inactivate the enzyme. The DFPH was stored in glass bottles at 4 °C. The degree of hydrolysis (DH) was used as an indicator for cleavage of peptide bond. Percent DH is defined as percent of total peptide bond cleaved. The degree of hydrolysis was calculated according to Adler-Nissen (1986).

Chemical composition

Dry weight of fish powders was determined after heating samples at 105 °C to constant weight, and ash content was determined after heating dried samples at 600 °C for 24 h. Total nitrogen content was determined using the Kjeldahl method. Crude protein was estimated by multiplying total nitrogen content by the factor 6.25. Crude fat was determined gravimetrically after Soxhlet extraction of dried samples with diethyl ether.

Biomass measurement

Biomass concentration was determined by filtration of all the culture medium through a Whatman filter paper. Filters were then washed with distilled water, and dried at 80 °C to constant weight.

Assay of lipolytic activity

Lipase activity was measured with olive oil as substrate, by continuous titration with NaOH 100 mM at 37 °C, pH 8.2 in a pH-stat. The enzymatic reaction was performed in a reaction medium containing: 250 μl bovine serum albumin, 20 ml distilled water and 10 ml of a 10% (v/v) emulsion of olive oil. The olive oil was emulsified by sonication of 10 ml of olive oil with 90 ml gum arabic (10%). Culture medium supernatant containing lipase (1–10 μl depending on the lipase activity) was added to the medium and the enzyme activity was determined.

One unit of lipase activity was defined as the amount of enzyme that released 1 μmol of free fatty acid per minute under assay conditions.

Results and discussion

Preparation of fish powders and fish protein hydrolysates

Different *Sardinella* substrates were prepared and used as nitrogen source: meat and whole fish powders (MFP and WFP), defatted meat–fish powder (DMFP), meat–fish protein hydrolysate (MFPH) and defatted meat–fish protein hydrolysate (DMFPH) (Figure 1). Table 1

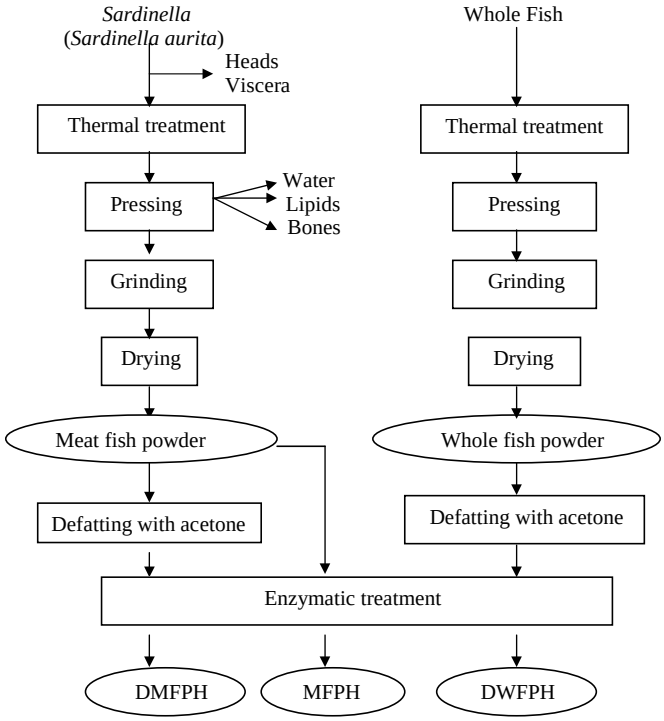

Figure 1. Flow diagrams for the preparation of fish powders and fish protein hydrolysates. DMFPH: defatting meat–fish protein hydrolysate; MFPH: meat–fish protein hydrolysate; DWFPH: defatting whole-fish protein hydrolysate.

Table 1. Chemical composition of fish powders (g/100 g product).

	Water	Protein $n \times 6.25$	Lipids	Ash
Whole Sardinella	1.3	64.3	11.4	11.7
Defatted whole Sardinella	1.5	70.5	4.5	12
Meat Sardinella	1.3	80.0	8.0	4.4
Defatted meat Sardinella	2.0	84.5	3.5	4.3

shows the chemical composition of the defatted and non-defatted fish powders.

As shown in Table 1, meat *Sardinella* contained higher protein content (80%) but lower ash and lipid content than whole fish powder. Elimination of heads, viscera and bones resulted in an increase in the protein content. The residual fat content of the meat material after treatment with acetone was about 3.5/100 g of products.

To obtain defatted fish protein hydrolysates, fish powders, obtained as shown in Figure 1, were first defatted with acetone then subjected to hydrolysis with proteases. Digestions were conducted at 60 °C for about 3 h.

Evidence of lipase production by R. oryzae *grown on different Sardinella preparations*

In order to test if fish preparations promote biomass and enzyme synthesis, lipase production by *R. oryzae* was assayed in R1 medium containing fish substrates as a nitrogen source. All cases were compared with a control medium containing soy peptone as nitrogen source (Figure 2).

When the strain was cultivated in media containing whole fish, meat–fish or defatted meat–fish powders as nitrogen source, lipase production was significantly low, about 15 U ml^{-1}. The lower production compared to that obtained with commercial peptone could be due to the use of complex organic nitrogen source (intact fish proteins). *R. oryzae* produces protease during the late of growth phase. In the early growth phase, the level of protease is very low and it's insufficient to release, from undigested fish proteins, free amino acids and small peptides essential for biomass production and lipase

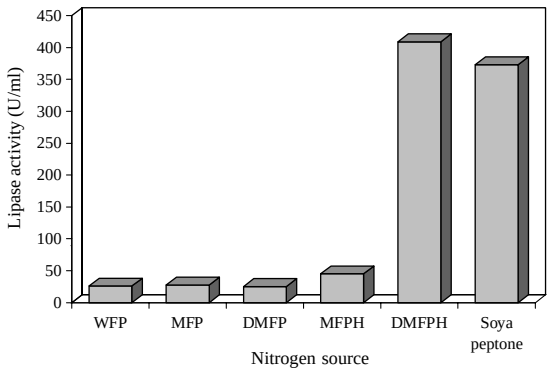

Figure 2. Effect of different *Sardinella* preparations on lipase production by *R. oryzae*. Cultures were conducted in R1 medium containing 40 g of fish substrate l^{-1} as the nitrogen source. WFP: whole-fish powder; MFP: meat–fish powder, DMFP: defatted meat–fish powder; MFPH: meat–fish protein hydrolysate; DMFPH: defatted meat–fish protein hydrolysate.

synthesis. Only proteolytic strains can grow in medium contains intact proteins (Battaglino *et al*. 1991). In a previous paper, Ellouz *et al*. (2001) have shown that protease synthesis was strongly induced when *Bacillus subtilis* strain was grown in medium containing only *Sardinella* heads and viscera powder.

Lipase activity was slightly increased when *Sardinella* protein hydrolysate, obtained from non-defatted meat–fish powder, was used as the nitrogen source but the enzyme production was still poor (about 45 U ml^{-1}), compared to that obtained with commercial peptone medium (Figure 2).

The lower lipase induction obtained in medium containing non-defatted FPH, could be due to the existence in lipid fraction, of a compound, that could repress enzyme synthesis. In order to study such hypothesis, DMFPH was tested for lipase production. The lipid content was reduced to 4% from 10.1% of raw *Sardinella*. Figure 2 shows that lipase synthesis is strongly enhanced (394 U ml^{-1}) and the enzyme activity is slightly greater than that obtained with commercial peptones as nitrogen source (373 U ml^{-1}). These results clearly indicated that lipase synthesis is affected by the composition of the medium.

As many hydrolytic enzymes, lipases are induced by substrates upon which they acted and repressed by their degradation products. Fatty acids have been shown to repress lipase synthesis in many lipolytic strains. With lipolytic strains, oil can be used as sole carbon source (Tan & Gill 1985) or as an inducer in complex media for the production of lipase (Dharmsthiti & Kuhasuntisuk 1998). The production of lipase by *Yarrowia lipolytica* 681 was enhanced significantly by olive or corn oil when used as both carbon and inducer sources. Corn and olive oil was found to increase the yield of extracellular lipase in other strains such as *Candida rugosa* (Benjamin & Pandey 1996) and *Aspergillus niger* (Hatzinikolaou *et al*. 1996). Recently, Miranda *et al*. (1999) have reported the production of lipase by a Brazilian strain of *Penicillium citrinum* using an industrial residue. Cultivated in a medium containing a residue from a vegetable oil refinery as carbon source and ammonium nitrate as nitrogen source, lipase production by the strain was higher than that obtained with olive oil.

Effect of culture media on lipase production

Since defatted meat fish protein hydrolysate was the best nitrogen source for lipase synthesis by *R. oryzae*, the effect of its concentration on enzyme production was studied. As shown in Table 2, lipase activity reached a maximum value at 40 g l^{-1} (394 U ml^{-1}). At larger concentrations than 40 g l^{-1}, lipase production decreased, although biomass increased continuously. The product yield, estimated as lipase produced per gram of nitrogen substrate added or biomass produced, reached maximum values of 15.8×10^3 and 36×10^3 UI g^{-1}, respectively, at 24 g of DMFPH l^{-1}. Furthermore, when DMFPH concentration increased the product yields decreased. Thus concentration of 24 g l^{-1} seems to be adequate.

Table 2. Effect of DMFPH concentrations on lipase production by *R. oryzae*.

DMFPH (g l^{-1})	8	16	24	40	56
Lipase activity, U ml^{-1}	23	244	379	394	350
Biomass, g l^{-1}	5.4	8.6	10.5	14.1	18.7
Yield (10^3 U g^{-1} nitrogen added)	2.9	15.3	15.8	9.9	6.3
Yield (10^3 U g^{-1} biomass)	4.3	28.4	36.1	27.9	18.7

In order to test if DMFPH alone promotes biomass and lipase production, some components of R1 medium were selectively removed. Experiments were conducted with 24 g of DMFPH l^{-1}. Results presented in Table 3 show that the elimination of both glucose and salts reduced considerably the biomass and lipase formation compared to control medium, which contains all the constituents. However, when glucose was included in the medium containing only DMFPH, the strain exhibited a slightly greater lipase production than that obtained with control medium.

From the results shown in Table 3, it is shown that DMFPH supplies the strain with some minerals but glucose is needed, since elimination of the carbon source drastically reduced lipase synthesis. The maximum lipase activity (412 U ml^{-1}) and the highest specific yield (42.9 U mg^{-1} of dry cells) were achieved with 10 g glucose l^{-1}. Beyond 10 g of glucose l^{-1}, lipase activity decreased whereas biomass increased (Table 4).

Effect of the degree of hydrolysis on lipase production

In order to study the importance of the degree of hydrolysis on lipase synthesis, different FPH were prepared from defatted meat *Sardinella* by enzymic

Table 3. Effect of culture media on lipase production by *R. oryzae*.

	DMFPH + Glucose + Salts	DMFPH	DMFPH + glucose
Lipase activity (U ml^{-1})	379	35	412
Biomass (g l^{-1})	10.8	4.1	9.2

Cultures were operated in media containing 24 g of DMFPH l^{-1}.

Table 4. Effect of glucose concentrations on lipase production by *R. oryzae*.

Glucose (g l^{-1})	0	2	5	10	15
Lipase activity (U ml^{-1})	35	180	330	412	325
Biomass (g l^{-1})	3.4	6.7	8.3	9.6	12.9
Yield (10^3 U g^{-1} biomass)	10.3	26.9	39.7	42.9	25.2

Cultures were operated in media containing 24 g of DMFPH l^{-1} and different concentrations of glucose.

Table 5. Effect of the degree of hydrolysis on lipase production by *R. oryzae*.

	J21	MN	Neutrase	Alcalase
DH (%)	3–4	4–5	11–13	27–30
Relative activity (%)	55	60	80	100
Biomass (g l^{-1})	5.0	5.5	7.7	8.9
Final pH	7.0	6.9	7.4	7.5

treatment using various proteases: Alcalase, Neutrase, J21 and MN7 enzymes. As shown in Table 5, FPH treated with Alcalase showed an appreciably higher degree of hydrolysis (HD: 27–30%) than those obtained with Neutrase, J21 and MN7 enzymes. J21 has the lower proteolytic activity.

Table 5 shows results from the different hydrolysates. *R. oryzae* grew better and produced higher lipase activity with FPH obtained using Alcalase. This higher lipase induction could be due to the extensive hydrolysis of fish proteins, which resulted in a product containing free amino acids and low molecular-weight peptides, essential for microbial growth and metabolite synthesis.

Results presented here clearly indicated that the level of hydrolysis seems to play an important role in lipase synthesis.

Conclusion

In conclusion, the results obtained here clearly indicated that defatted *Sardinella* meat protein hydrolysate may be an excellent nitrogen source for the growth of *R. oryzae* and the production of lipase. By comparison with the results obtained with commercial peptone, there is a slightly improvement of lipase with fish media. The high lipase activity obtained with cheap fish meal clearly indicated that these substrates could be used in industrial fermentation processes.

Acknowledgements

This work was supported financially by Ministère de l'Enseignement Supérieur, de la Recherche Scientifique et de la Technologie, Tunisie.

References

Adler-Nissen, J. 1986 *Enzymic Hydrolysis of Food Proteins*. Barking, UK: Elsevier Applied Science Publishers. ISBN 0-85334386-1.

Battaglino, R.A., Huergo, M., Pilosof, A.M.R. & Bartholomai, G.B. 1991 Culture requirements for the production of protease by *Aspergillus oryzae* in solid state fermentation. *Applied Microbiology and Biotechnology* **35**, 292–296.

Benjamin, B. & Pandey, A. 1996 Optimization of liquid media for lipase production by *Candida rugosa*. *Bioresource Technology* **55**, 167–170.

Ben Salah, A., Fendri, K. & Gargouri, Y. 1994 La lipase de *Rhyzopus oryzae*: production, purification et caractéristiques biochimiques. *Revue Française des Corps Gras* **5**, 133–136.

Clausen, E., Gildberg, A. & Raa, J. 1985 Preparation and testing of an autolysate of fish viscera as growth substrate for bacteria. *Applied and Environmental Microbiology* **50**, 1556–1557.

Coello, N., Brito, L. & Nonus, M. 2000 Biosynthesis of L-lysine by *Corynebacterium glutamicum* grown on fish silage. *Bioresource Technology* **73**, 221–225.

De la Broise, D., Dauer, G., Gildberg, A. & Guérard, F. 1998 Evidence of positive effect of peptone hydrolysis rate on *Escherichia coli* culture kinetics. *Journal of Marine Biotechnology* **6**, 111–115.

Dharmsthiti, S. & Kuhasuntisuk, B. 1998 Lipase from *Pseudomonas aeruginosa* LP602: biochemical properties and application for wastewater treatment. *Journal of Industrial Microbiology and Biotechnology* **21**, 75–80.

Dufossé, L., De la Broise, D. & Guérard, F. 1997 Review: fish protein hydrolysates as nitrogen sources for microbial growth and metabolite production. In *Recent Research Developments in Microbiology*, vol. 1. pp. 365–381. Trivandrum, India: Research Sign Post Publ.

Dufossé, L., De la Broise, D. & Guérard, F. 2001 Evaluation of nitrogenous substrates such as peptones from fish: a new method based on Gompertz modeling of microbial growth. *Current Microbiology* **42**, 32–38.

Ellouz, Y., Bayoudh, A., Kammoun, S., Gharsallah, N. & Nasri, M. 2001 Production of protease by *Bacillus subtilis* grown on sardinella heads and viscera flour. *Bioresource Technology* **80**, 49–51.

Gandhi, N.N. 1997 Applications of lipase. *Journal of the American Oil Chemists' Society* **74**, 534–621.

Gildberg, A., Batista, I. & Ström, E. 1989 Preparation and characterization of peptones obtained by two-step enzymatic hydrolysis of whole fish. *Biotechnology and Applied Biochemistry* **11**, 413–423.

Haltrich, D., Laussamayer, B. & Steiner, W. 1994 Xylanase formation by *Sclerotium rolfsii*: effect of growth substrates and development of a culture medium using statistically designed experiments. *Applied Microbiology and Biotechnology* **42**, 522–530.

Hatzinikolaou, D.G., Macris, J.B., Christakopoulos, P., Rekos, D., Kolisis, F.N. & Fountoukidis, G. 1996 Production and partial characterization of extra-cellular lipase from *Aspergillus niger*. *Biotechnology Letters* **18**, 547–552.

Iwai, M. & Tsujisaka, Y. 1984 Fungal lipases. In *Lipases*, eds. Borgström, B. & Brockman, H.L. (pp. 443–449) Amsterdam: Elsevier. ISBN 0-44480526-5.

Kristinsson, H.G. & Rasco, B.A. 2000 Fish protein hydrolysates: production, biochemical, and functional properties. *CRC Critical Reviews in Food Science and Nutrition* **40**, 43–81.

Miranda, O.A., Salgueiro, A.A., Pimentel, M.C.B., Lima Filho, J.L., Melo, E.H.M. & Duran, N. 1999 Lipase production by a Brazilian strain of *Penicillium citrinum* using an industrial residue. *Bioresource Technology* **69**, 45–147.

Nakashima, T., Kyotani, S., Izumoto, E. & Fukuda, H. 1990 Cell aggregation as a trigger for enhancement of intracellular lipase production by a *Rhizopus* species. *Journal of Fermentation Bioengineering* **70**, 85–89.

Rapp, P. 1995 Production, regulation, and some properties of lipase activity from *Fusarium oxysporum* f. sp. Vasinfectum. *Enzyme and Microbial Technology* **17**, 832–838.

Reed, G. 1975 Microbial lipases. In *Enzymes in Food Processing*. ed. Reed, G. (pp. 208–214). New York: Academic Press. ISBN 0-12-584852-8.

Reed Hamer, B. & West, T.P. 1994 Effect of complex nitrogen sources on pullulan production relative to carbon source. *Microbios* **80**, 83–90.

Reissbrodt, R., Beer, W., Muller, R., Claus, H. 1995 Characterization of casein peptones by HPLC profiles and microbiological growth parameters. *Acta Biotechnologica* **15**, 223–231.

Rodriguez-Estrada, M.T., Chung, S. & Chinachoti, P. 1994 Solids extraction of cod frame and effects on ultrafiltration of the aqueous extract. *Journal of Food Science* **59**, 799–803.

Saad, R.R. 1995 Production of lipase from *Aspergillus tamarii* and its compatibility with commercial detergents. *Folia Microbiologica* **40**, 263–266.

Salleh, A.B., Musani, R., Basri, M., Ampon, K., Yunus, W.M.Z. & Razak, C.N.A. 1993 Extra- and intra-cellular lipases from a thermophilic *Rhizopus oryzae* and factors affecting their production. *Canadian Journal of Microbiology* **39**, 978–981.

Singh, G., Kothari, R.M., Sharma, R.K. & Ramamurthy, V. 1995 Enhancement of *Spirulina* biomass productivity by a protein hydrolysate. *Applied Biochemistry and Biotechnology* **50**, 285–290.

Tan, H.G. & Gill, C.O. 1985 Effect of culture conditions on batch growth of *Pseudomonas fluorescens* on olive oil. *Applied Microbiology and Biotechnology* **23**, 27–32.