ISSN 1330-9862
(FTB-1504)

original scientific paper

Production and Partial Purification of a Neutral Metalloprotease by Fungal Mixed Substrate Fermentation

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Received: May 5, 2005
Accepted: June 17, 2005

Summary

Five strains of fungi belonging to *Aspergillus* sp. were evaluated by casein agar plate assay and a wheat bran-based solid-state fermentation for selecting a neutral protease-producing culture. Based on the results, *A. oryzae* NRRL 2217 was selected for further studies. Sixteen different agro-industrial residues were evaluated for their potential to serve as a substrate for neutral protease production by this fungal strain. Results showed that a combination of coconut oil cake and wheat bran in the mass ratio of 1:3 was the best substrate for enzyme production. Various process parameters influencing protease production including fermentation time, initial moisture content, and fermentation temperature were optimised. The medium was supplemented with different nutrients in the form of organic and inorganic nitrogen and carbon sources. Supplementation of chitin increased the enzyme production significantly. Ammonium nitrate as inorganic nitrogen supplement slightly enhanced enzyme production. No organic nitrogen supplement was effective enhancer of enzyme production. Fermentation was performed under optimised conditions (initial moisture content V/m = 50 %, temperature 30 °C, 48 h). Partial purification of the enzyme resulted in a 3-fold increase in the specific activity of the enzyme. The partially purified enzyme was characterised by various features that govern the enzyme activity such as assay temperature, assay pH and substrate concentration. The effect of various metal ions and known protease inhibitors on the enzyme activity was also studied. The enzyme was found to be stable in pH range 7.0–7.5, and at temperature of 50 °C for 35 min. By the activating effect of divalent cations (Mg^{2+}, Ca^{2+}, Fe^{2+}) and inhibiting effect of certain chelating agents (EGTA, EDTA), the enzyme was found to be a metalloprotease.

Key words: neutral protease, solid-state fermentation, *Aspergillus*, agro-industrial residues

Introduction

Proteases are one of industrially most important enzymes. The main sources of these enzymes were animals (*e.g.* calf stomach) and plants (*e.g.* pineapple, fig, papaya). Due to the irregular production associated with plant sources and large number of moral and ethical issues related to animal sources, microbial sources have occupied an important place in the production of all the three major types of proteases – acidic, neutral, and alkaline (*1*). Microorganisms, especially fungi, owing to their GRAS (Generally Regarded As Safe) nature, have

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now become popular, especially with respect to enzyme application in the food industries (*2*). Fungal neutral proteases are the most important component of commercial fungal protease preparations, which have applications in baking, food processing, animal feeds, and pharmaceutical industries. *Aspergillus oryzae* is the predominant fungal source of the enzyme (*3,4*). Its affinity for hydrophobic amino acids provides an advantage for its utilization as a debittering agent.

Enzyme production by fermentation can be carried out by both submerged fermentation (SmF) and solid-state fermentation (SSF). SSF has been established as a superior technique (as compared to submerged fermentation) for the production of enzymes (*5,6*). Solid-state fermentations involve microbial modification of a solid, undissolved substrate in which microbial cultures are grown on a moist solid with little or no free water, although capillary water may be present (*7*). The product can be recovered in highly concentrated form as compared to those obtained by submerged fermentations. Traditionally, synthetic substrates were used for fermentations, which are now being largely replaced by agro and agro-industrial by-products. These not only provide a natural substrate for fungal growth and fermentation, they also result in improved value of these agro-industrial residues (*8*). In this study, SSF of agro by-products was carried out using a GRAS strain of a fungus to produce a neutral protease with potential use as a food enzyme.

The proteases constitute a very large and complex group of enzymes, which differ in properties such as substrate specificity, active site and catalytic mechanism, pH and temperature optima, and stability profile. Studies relating to such properties of these proteases are imperative for the successful application of these enzymes in their respective industry (*9,10*). The enzyme produced by SSF is hence characterised by various features that influence enzyme activity such as optimum temperature, optimum pH, effect of metal ions, *etc.*

Material and Methods

Microorganisms and maintenance of culture

Five strains of *Aspergillus* sp. (*A. oryzae* NRRL 1989, 2217 and 3485, and *A. sojae* NRRL 6271 and 1988) were used in this study. The fungi were grown and maintained on potato dextrose agar (PDA) slants. Spore suspension of 10^6 spores/mL in 0.1 % Tween 80, produced from one-week-old fully sporulated slant, was used for inoculation.

Substrates

All the substrates used in the study were obtained from local market in Trivandrum, except olive oil cake (which was obtained from Greece), palm kernel cake (from Malaysia), groundnut oil cake (from Rajkot, India), sugarcane bagasse (from Dehradun, India), and cassava bagasse (from Salem, India).

Screening of neutral protease producers by plate assay

Strains were screened for neutral protease production using a qualitative plate medium at pH=7.0 in Petri plates (90 mm diameter) containing (in %): glycerol 0.5, casein 1, yeast extract 0.3, NaCl 0.5, and agar 2. The medium was inoculated with fungal spores (0.1 mL of spore suspension) and incubated at 30 °C for 72 h. The plates were then stained with Coomassie Brilliant Blue R-250 for 2 h followed by destaining overnight to observe the hydrolysis zone by neutral protease. The radius of the hydrolysis zone was measured.

Screening of neutral protease producers by SSF

Wheat bran was used as the substrate for screening the most potent proteolytic fungus by a quantitative assay in SSF. A mass of 5 g of the dry substrate was introduced in 250-mL Erlenmeyer flask and supplemented with 4 mL of mineral medium containing (in %): NH_4NO_3 0.5, KH_2PO_4 0.2, $MgSO_4 \cdot 7\,H_2O$ 0.1, and NaCl 0.1. The contents were sterilized by autoclaving at 121 °C for 15 min. After cooling, the flasks were inoculated with 1 mL of spore suspension and incubated at 30 °C for 72 h unless otherwise specified. All experiments were carried out in two sets. The results shown are average values ± s.d.

Screening of substrates

Sixteen agro-industrial residues were screened for protease production by SSF. These were sesame oil cake (SOC), olive oil cake (OOC), mustard oil cake (MOC), groundnut oil cake (GOC), cotton seed oil cake (CSOC), palm kernel cake (PKC), spent brewing grains (SBG), rice bran (RB), rice husk (RH), cassava bagasse (CB), sugarcane bagasse (SB), prawn shell powder (PSP), coconut oil cake (COC), tamarind seed chips (TSC), jackfruit seed powder (JSP), and wheat bran (WB).

Optimisation of process parameters

Various process parameters influencing protease production such as fermentation time (0, 24, 48, 72, 96, 120, 144 and 168 h), initial moisture content (44.44, 47.36, 50, 52.38 and 54.54 %), and fermentation temperature (16, 23, 30, 37 and 44 °C) were optimised. The medium was supplemented with different carbon sources (cellulose, chitin, dextrose, inulin, lactose, mannitol, maltose, sorbitol, hydrolysed and soluble starch, sucrose and xylose), different organic nitrogen sources (beef extract, casein, corn steep liquor, corn steep solids, malt extract, peptone, tryptone and yeast extract) and with different inorganic nitrogen sources (ammonium bicarbonate, ammonium dihydrogen phosphate, diammonium hydrogen phosphate, ammonium nitrate, potassium nitrate, sodium nitrate, ammonium sulphate and ammonium chloride). SSF was also performed under optimised conditions.

Enzyme extraction

A known quantity (5 g) of the fermented material was mixed with 50 mL of Tween 80 solution in distilled water and homogenized by mixing in a rotary shaker at 180 rpm for 1 h. This homogenized substrate was centrifuged at 8000 x *g* at 4 °C for 15 min. The supernatant obtained was used for analytical studies.

Analytical methods

Assay for neutral protease

The neutral protease activity in the crude enzyme extract was assayed using casein as substrate at pH=7.0 (0.2 M phosphate buffer) and 60 °C (*11*). After incubation for 10 min, the reaction was stopped using trichloroacetic acid and the amount of the liberation of amino acids was quantified by measuring the intensity of the blue colour at 660 nm using a tyrosine standard (*12*). The colour was developed by adding Folin-Ciocalteau's phenol reagent to the TCA-precipitate-free supernatant. One unit of enzyme activity is defined as the amount of enzyme that liberates 1 μmol of tyrosine from the substrate per minute per gram of dry substrate under the assay conditions.

Estimation of total soluble protein

The total soluble protein content was estimated by Lowry's method (*12*) using BSA as standard.

Partial purification of the enzyme

The supernatant was separated into three fractions based on the percentage of saturation of ammonium sulphate. The precipitated proteins were separated by centrifugation at 10 000 x *g* at 4 °C for 15 min. These were dissolved in phosphate buffer (pH=7.0) and stored at 4 °C. The precipitated enzyme samples were dialyzed against phosphate buffer (pH=7.0) at 4 °C for 24 h with buffer changes at regular intervals.

Characterisation of the enzyme

Temperature optimum

The influence of temperature on the activity of neutral protease was studied by incubating the reaction mixture at different temperatures (30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90 and 95 °C).

Effect of substrate concentration

The effect of substrate concentration on the activity of neutral protease was studied by using different concentrations of casein (5, 10, 15, 20, 25 and 30 mg/mL).

Effect of activators/inhibitors

Different metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+}) and known protease inhibitors (PMSF, EDTA, EGTA, DTT and SDS) at a concentration of 0.1 M were used to study their effect on enzyme activity.

Stability at optimum temperature

The reaction mixture was incubated at its optimum temperature for different time intervals (5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 min) to study the stability of the enzyme.

pH optimum

The pH optimum of the neutral protease enzyme was determined by using buffer solutions of different pH (phosphate buffer 6.0, 6.5, 7.0, 7.5 and 8.0, and Tris-HCl buffer 8.5 and 9.0).

Results and Discussion

Screening of neutral protease producers

The casein agar plate assay and wheat bran SSF revealed that *A. oryzae* NRRL 1989 and 2217 were comparatively better producers of neutral protease. The hydrolysis zone produced on the casein agar could be related to the amount of protease produced by the fungus (*13*). But some exceptions have been reported such as the protease produced by *Bacillus licheniformis*, which produces very narrow zones of hydrolysis on casein agar plates inspite of large enzyme production by submerged culture (*14*). As shown in Table 1, while *A. oryzae* NRRL 2217 produced the largest hydrolysis zone on the plate medium, *A. oryzae* NRRL 1989 gave the highest enzyme units in SSF. Hence, these two strains were selected for substrate screening.

Table 1. Neutral protease production by different *Aspergillus* sp. on wheat bran-based SSF and on plate medium, measured respectively as enzyme produced and as hydrolysis zone produced on casein agar. Standard deviation for all values is < ± 5 %

Microorganism	Protease activity	Zone of hydrolysis
	U/g	mm
A. oryzae NRRL 1989	7.79	1.6
A. oryzae NRRL 3485	5.61	–
A. oryzae NRRL 2217	7.00	2.1
A. sojae NRRL 6271	5.59	1.2
A. sojae NRRL 1988	5.86	1.0

Screening of substrates

Fig. 1 revealed that both strains of *A. oryzae* gave maximum enzyme units per gram of dry substrate (U/g) on both COC and WB, though JSP also seemed to be a good substrate for neutral protease production by *A. oryzae* NRRL 2217. Subsequently, with a view to select one strain for production studies, different combinations of COC and WB (mass ratio of 1:0, 0:1, 1:1, 1:2, 1:3, 1:4, 2:1, 3:1 and 4:1) were used as substrates for *A. oryzae* NRRL 2217 and NRRL 1989. As evident from Fig. 2, *A. oryzae* NRRL 2217 was the highest enzyme producer on COC:WB in mass ratio of 1:3. Mixed substrate (COC and

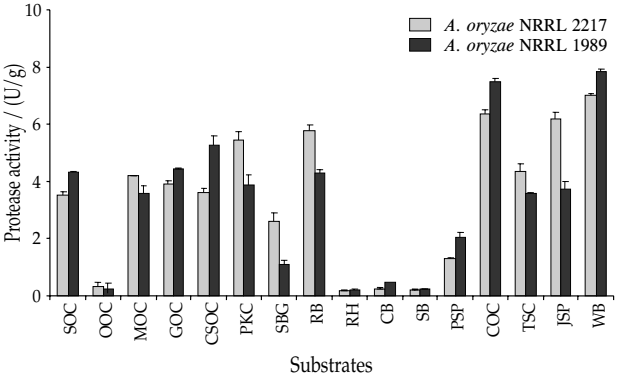


Fig. 1. Screening of agro-industrial residues as fermentation substrates for the production of neutral protease

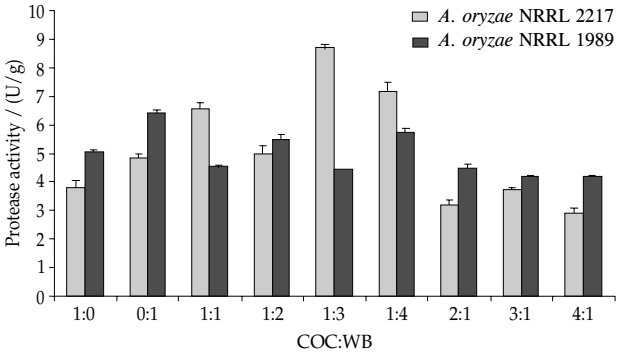


Fig. 2. Varying mass ratios of COC and WB as substrates for neutral protease production

WB) in the mass ratio of 1:2 was also found most suitable for lipase production by *Candida rugosa* (*15*). Thus, with this strain (*A. oryzae* NRRL 2217), process optimisation for neutral protease production was carried out using the mixed substrate.

Optimisation of process parameters

Any fermentation process is governed by a large number of physical, chemical, and biological factors. However, the parameters that largely impact enzyme production by SSF are fermentation time, initial moisture content, and fermentation temperature. Nutrient supplements such as carbon and nitrogen (both organic and inorganic) can also affect enzyme production. Fig. 3 shows that maximal enzyme production occurs at 48 h. The subsequent decrease in enzyme activity with increasing fermentation time could possibly be due to cessation of production, as enzymes are primary metabolites, and it could also be due to enzyme deactivation. Initial moisture content and temperature play crucial role in enzyme production by SSF (*16*). Initial moisture content usually varies depending on the type of substrate, and here, any increase or decrease of the moisture was not favourable for enzyme production (Table 2). Likewise, since the fungus was mesophilic in nature, variation in temperature above or below its optimum (30 °C) led to a decline in enzyme production (Table 2).

The solid substrates used for fermentation, being agro by-products, might lack in nutrients and hence external supplementation might positively contribute to enzyme production. But, as revealed from Table 3, none of the inorganic (except ammonium nitrate, though not significantly) or organic nitrogen supplements enhanced enzyme production in contrast to many reports that a nitrogen-rich media favoured protease production (*17*). This might be due to the high protein content of COC substrate, which supplies the required nitrogen.

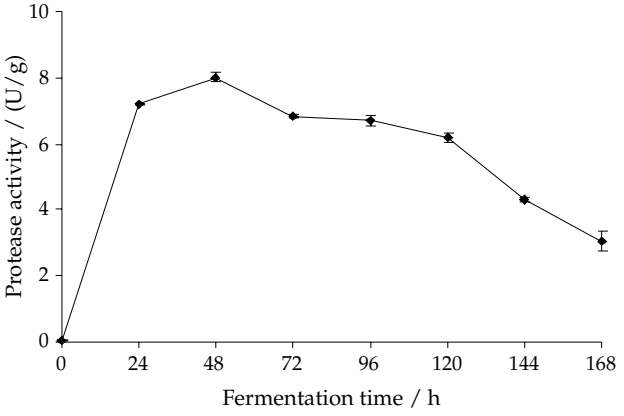


Fig. 3. Production profile of neutral protease with respect to time

Table 2. Effect of initial moisture content and fermentation temperature on neutral protease production. Standard deviation for all values is < ± 5 %

Initial moisture content	Protease activity	Fermentation temperature	Protease activity
%	U/g	°C	U/g
44.4	6.30	16	0.00
47.4	6.84	23	4.85
50.0	7.46	30	7.05
52.4	5.15	37	2.66
54.5	4.59	44	0.04

Table 3. Effect of different inorganic and organic nitrogen supplements on the production of neutral protease. Standard deviation for all values is < ± 5 %

Inorganic nitrogen supplement	Protease activity	Organic nitrogen supplement	Protease activity
	U/g		U/g
NH_4HCO_3	7.57	Beef extract	5.64
$NH_4H_2PO_4$	5.18	Casein	5.37
$(NH_4)_2HPO_4$	7.46	Corn steep liquor	5.61
NH_4NO_3	8.60	Corn steep solids	7.56
KNO_3	7.19	Malt extract	5.20
$NaNO_3$	6.10	Peptone	6.56
$(NH_4)_2SO_4$	6.95	Tryptone	5.65
NH_4Cl	5.55	Yeast extract	6.43
Control	8.00	Control	8.44

Fig. 4 shows that chitin as a carbon source seemed to have a noticeable impact on enzyme production. This might be due to the proteolytic activity of some chitinases. However, there was no detectable level of chitinase in the enzyme extract (data not shown).

Partial purification of the enzyme

Partial purification of neutral protease by ammonium sulphate precipitation, followed by dialysis, resulted in about 3-fold increase in the specific activity (data not shown).

Characterisation of the enzyme

Effect of temperature

Fig. 5 gives the temperature curve for the neutral protease. An initial increase in temperature of up to 50 °C increased the rate of the enzyme-catalyzed reaction

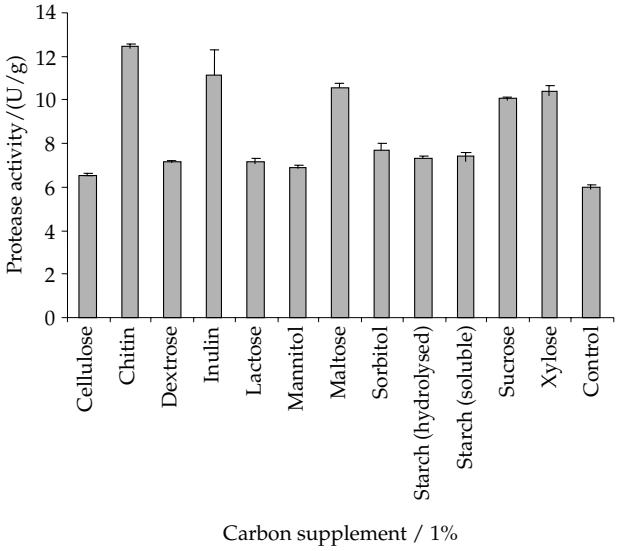


Fig. 4. Effect of different carbon supplements on the production of neutral protease

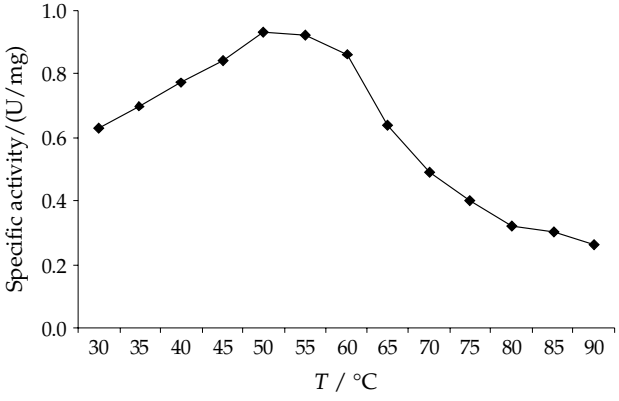


Fig. 5. Temperature curve of the partially purified neutral protease

due to the increase in number of collisions between the reacting molecules. But, the enzyme probably got denatured on exposure to temperatures higher than its optimum and thereafter, steadily lost its activity.

The partially purified enzyme was stable at its optimum temperature (50 °C) for up to 35 min (Fig. 6), beyond which it gradually lost its activity. Increase in incubation time of up to 35 min resulted in a gradual increase in the enzyme activity. This could be accounted for by the increasing quantity of substrate consumed with time and hence a concomitant increase in the amount of product liberated. The temperature and stability profile of the enzyme suggested that the enzyme was not thermostable. Since most food industries such as baking, brewing, *etc.* that utilise proteases require their processes to be carried out at temperatures around 50–60 °C, this enzyme could be of potential application as a food-processing agent.

Effect of substrate concentration

The Lineweaver-Burk (LB) plot (Fig. 7) for the proteolytic reaction of casein revealed that the V_{max} of the reaction was 1.85 μM/min. The Michaelis-Menten constant (K_m) was 2.94. The reaction rate remained constant beyond a substrate concentration of 20 mg/mL of casein (data not shown).

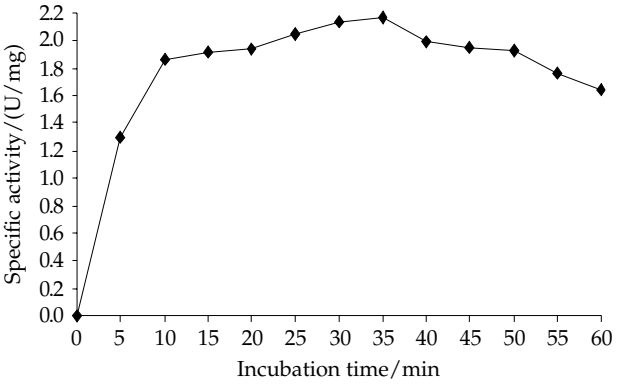


Fig. 6. Stability profile of neutral protease at its optimum temperature

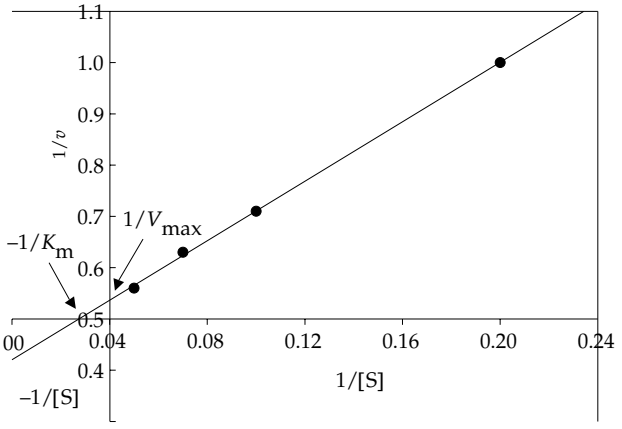
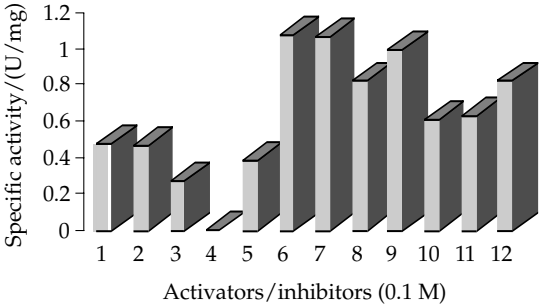


Fig. 7. Lineweaver-Burk plot for neutral protease under varying substrate (casein) concentrations (5–30 mg/mL) indicating the K_m and V_{max} values

Effect of activators/inhibitors

Fig. 8 shows that the enzyme was a metalloprotease. It was activated by certain metal ions such as Ca^{2+}, Mg^{2+} and Fe^{2+}, and inhibited by chelating agents such as EGTA and EDTA. The enzyme was also fairly susceptible to inhibitors such as PMSF, SDS and DTT (Fig. 8).



1 – PMSF; 2 – EDTA; 3 – EGTA; 4 – DTT; 5 – SDS; 6 – $CaCl_2$; 7 – $MgSO_4$; 8 – $MnSO_4$; 9 – $FeSO_4$; 10 – $CuSO_4$; 11 – $ZnSO_4$; 12 – Control

Fig. 8. Effect of various metal ions/protease inhibitors on neutral protease activity

Effect of pH

The enzyme exhibited maximum activity in the pH range of 7.0–7.5 (Fig. 9). The activity of neutral proteases generally decreases towards the alkaline side. But in this

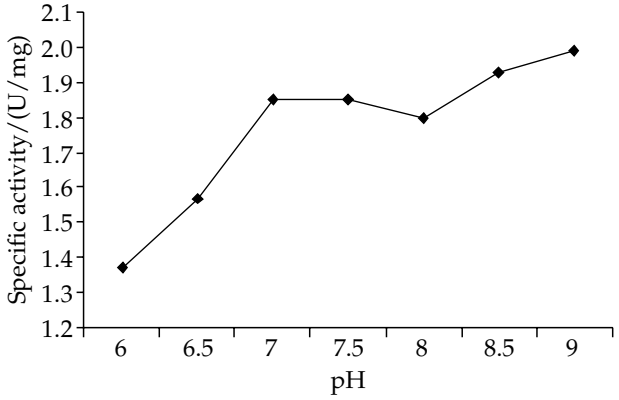


Fig. 9. pH profile of the partially purified neutral protease

case, a second increase in activity (with increase in pH) was observed, which was probably due to the presence of co-production of alkaline protease (data not shown).

Conclusions

Food enzymes such as proteases have been traditionally produced by submerged fermentation of substrates such as corn flour, soybean meal, products of protein-rich legumes such as groundnuts, *etc.* Submerged fermentation is often viewed disadvantageously owing to its high operation cost. Enzyme production by SSF using agro by-products not only brings down the cost of production (both of fermentation and downstream processing), but it also provides an alternative path for the effective and productive utilisation of such nutrient-rich agro residues. The GRAS nature of the fungi also adds up as an advantage in using the fungal products as food processing agents, and hence, promotes the usage of fungal SSF as the method of choice for the production of these proteolytic biocatalysts. The above study focussed on the use of a fungus to convert agro-industrial by-products to a value-added product, an enzyme. From the results, it could be inferred that neutral protease produced through SSF of the mixed substrate (COC+WB) by *A. oryzae* NRRL 2217 could possibly find useful application in food industries.

Acknowledgements

The authors thank CSIR, India for financial support under the network Task Force project (CMM 0006). A.S. gratefully acknowledges a Junior Research Fellowship from CSIR, New Delhi, India.

References

1. M.B. Rao, A.M. Tanksale, M.S. Ghatge, V. Deshpande, Molecular and biotechnological aspects of microbial proteases, *Microbiol. Mol. Biol. Rev. 62* (1998) 597–635.
2. A. Pandey, Recent process developments in solid-state fermentation, *Process Biochem. 27* (1992) 109–117.
3. T. Nakadai, S. Nasuno, N. Iguchi, The action of peptidases from *A. oryzae* in digestion of soybean proteins, *Agric. Biol. Chem. 36* (1972) 261–268.
4. T. Nakadai, S. Nasuno, N. Iguchi, Purification and properties of alkaline proteinase from *A. oryzae*, *Agric. Biol. Chem. 37* (1973) 2685–2694.
5. A. Pandey, C.R. Soccol, J.A. Rodriguez-Leon, P. Nigam: *Solid-State Fermentation in Biotechnology*, Asiatech Publishers Inc., New Delhi (2001).
6. C. Sandhya, A. Sumantha, G. Szakacs, A. Pandey, Comparative evaluation of neutral protease production by *Aspergillus oryzae* in submerged and solid state fermentation, *Process Biochem. 40* (2005) 2689–2694.
7. R.E. Mudgett: Solid State Fermentations. In: *Manual of Industrial Microbiology and Biotechnology*, A.L. Demain, N.A. Solomon (Eds.), ASM Press, Washington (1986) pp. 66–83.
8. A. Pandey, C.R. Soccol, D.A. Mitchell, New developments in solid-state fermentation: I. Bioprocesses and products, *Process Biochem. 35* (2000) 1153–1169.
9. C. Sandhya, A. Sumantha, A. Pandey: Proteases. In: *Enzyme Technology*, A. Pandey, C. Webb, C.R. Soccol, C. Larroche (Eds.), Asiatech Publishers Inc., New Delhi (2004) pp. 312–325.
10. S. Germano, A. Pandey, C.A. Osaku, S.N. Rocha, C.R. Soccol, Characterization and stability of proteases from *Penicillium* sp. produced by solid-state fermentation, *Enzyme Microb. Technol. 32* (2003) 246–251.
11. L. Keay, B.S. Wildi, Proteases of genus *Bacillus*. I. Neutral proteases, *Biotechnol. Bioeng. 12* (1970) 179–212.
12. O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with Folin phenol reagent, *J. Biol. Chem. 193* (1951) 265–275.
13. A.B. Vermelho, M.N.L. Meirelles, A. Lopes, S.D.G. Petinate, A.A. Chaia, M.H. Branquinha, Detection of extracellular proteases from microorganisms on agar plates, *Mem. Inst. Oswaldo Cruz, 91* (1996) *http://www.bioline.org.br*
14. K. Aunstrup: Industrial Production of Microbial Enzymes. In: *Industrial Aspects of Biochemistry*, B. Spencer (Ed.), Federation of European Biochemical Societies (1974) pp. 23–46.
15. S. Benjamin, A. Pandey, Coconut cake – A potent substrate for the production of lipase by *Candida rugosa* in solid state fermentation, *Acta Biotechnol. 17* (1997) 241–251.
16. A. Pandey, Solid-state fermentation, *Biochem. Eng. 13* (2003) 81–84.
17. K. Aunstrup: Proteinases. In: *Microbial Enzymes and Bioconversions: Economic Microbiology, Vol. 5*, A.H. Rose (Ed.), Academic Press, London (1980) pp. 49–114.

Proizvodnja i djelomična purifikacija neutralne metaloproteaze dobivene fermentacijom funga na miješanom supstratu

Sažetak

Ispitano je pet sojeva *Aspergillus* sp. pokusima na agaru s kazeinom i fermentacijom na čvrstoj podlozi od pšeničnih mekinja kako bi se odabrala kultura za proizvodnju neutralne proteaze. Na osnovi dobivenih rezultata odabran je soj *A. oryzae* NRRL 2217 za daljnja ispitivanja. Ispitano je šesnaest različitih agroindustrijskih otpadaka kao potencijalni supstrat za taj fungalni soj pri proizvodnji neutralne proteaze. Nađeno je da je najbolji supstrat za proizvodnju enzima kombinacija pogače kokosova ulja i pšeničnih mekinja u masenom omjeru 1:3. Optimirani su vrijeme fermentacije, početni udjel vlage i temperatura koji utječu na proizvodnju proteaze. Podloga je bila obogaćena raznim hranjivim tvarima u obliku organskih i anorganskih izvora dušika i ugljika. Dodatak hitina znatno je povećao proizvodnju enzima. Amonijev nitrat, kao anorganski dodatak dušika, malo je povećao proizvodnju enzima. Nijedan organski dodatak dušika nije poboljšao proizvodnju enzima. Tijekom 48 h fermentacija je provedena pod otimalnim uvjetima (početni udjel vlage V/m = 50 % i temperatura 30 °C). Parcijalno pročišćeni enzim imao je 3 puta veću specifičnu aktivnost, a njegova su svojstva bila određena prema temperaturi, pH i koncentraciji supstrata. Proučen je i utjecaj raznih iona metala i poznatih inhibitora proteaze na enzimsku aktivnost. Utvrđeno je da je enzim stabilan u području pH od 7,0 do 7,5, pri temperaturi od 50 °C tijekom 35 min. Enzim je metaloproteaza jer je aktiviran divalentnim kationima (Mg^{2+}, Ca^{2+}, Fe^{2+}), a inhibiran kelatnim agensima kao EGTA i EDTA.