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Process Biochemistry 40 (2005) 1131–1136

PROCESS BIOCHEMISTRY

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Alkaline protease production by a soil isolate of *Beauveria felina* under SSF condition: parameter optimization and application to soy protein hydrolysis

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Received 17 January 2004; accepted 31 March 2004

Abstract

Alkaline protease activity of a soil isolate of *Beauveria felina* for soy protein hydrolysis was explored and compared with *Aspergillus oryzae* NCIM 649, a known alkaline protease producer, under solid substrate fermentation (SSF) condition. The parameters affecting alkaline protease production under SSF condition were optimized. A maximum protease activity of ~20,000 U/g initial dry substrate (IDS) was obtained from *Beauveria felina* grown for 7 days on wheat bran moistened with M-9 solution (pH 7.0) and 120% initial moisture content. As compared to *Aspergillus oryzae* NCIM 649, *Beauveria felina* showed about two times higher alkaline protease production under optimized conditions. The results demonstrated the potential of *Beauveria felina* isolate as a source of alkaline protease for commercial application to soy protein hydrolysis.

Keywords: Alkaline protease; *Beauveria felina*; *Aspergillus oryzae* NCIM 649; Solid substrate fermentation; Soy protein hydrolysis

1. Introduction

Microorganisms represent an excellent source of proteases owing to their broad biochemical diversity and susceptibility to genetic manipulation [1]. Alkaline proteases find the single largest applications among the industrial proteases as an active ingredient of laundry detergents, in tannery, food and chemical industry, medical formulations, recovery of silver from X-ray films, etc. [2]. Various species of *Bacillus*, *Streptomyces* and *Aspergillus* have been extensively studied organisms for the production of alkaline proteases [2,3]. *Trichoderma* [4], *Rhizopus* [5] and *Penicillium* [6] represent some other alkaline protease producing genera among fungi. Although the role of alkaline protease as one of the virulence factors is well established in the case of *Beauveria* [7–9], this genus has not been explored for production of alkaline protease for commercial applications. Moreover, most of the alkaline protease production studies with *Beauveria* have been carried out under submerged fermentation (SmF) conditions.

There has been a strong incentive towards the use of soy protein and its products for varied applications on account of its large availability as soy flakes, a by-product of oil processing industries and excellent nutritional profile [10]. However, the functional and organoleptic properties of soy protein could be improved by enzymic modification [11]. Earlier, we have reported the hydrolysis of soy protein using alkaline protease of *Penicillium* sp. grown under solid substrate fermentation (SSF) conditions [12]. The present paper reports the optimization of parameters for the production of alkaline protease by a soil isolate identified as *Beauveria felina* under SSF conditions and its application to soy protein hydrolysis. It also compares the production of alkaline protease of *B. felina* with *Aspergillus oryzae* NCIM 649, a known alkaline protease producing fungus.

2. Materials and methods

2.1. Chemicals

Analytical grade chemicals of Hi-Media and E. Merck were used for the present study. Soy protein isolate was

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doi:10.1016/j.procbio.2004.03.006

prepared from defatted soy flour following the method described earlier with some modifications [13]. The procedure involved the preparation of a homogenous suspension of defatted soy flour in 10 volumes of distilled water, adjusting the pH to 8.5 and heating to 60 °C for 30 min. After cooling, the suspension was centrifuged at 10,000 rpm for 15 min; the supernatant was collected and adjusted to a pH of 4.5 with 2N HCl. The precipitated protein was filtered, washed with 50% ethanol, oven dried at 45 °C and ground to fine powder.

2.2. Microorganisms

A number of fungi were isolated from soil around soymeal manufacturing industry on modified Czapeck-Dox (CD) agar containing ($g\,l^{-1}$): sucrose, 10.0; $NaNO_3$, 1.0; K_2HPO_4, 1.0; $MgSO_4{\cdot}7H_2O$, 0.5; KC1, 0.5; $FeSO_4{\cdot}7H_2O$, 0.01; soy protein isolate, 1.0; agar, 20.0; pH adjusted to 7.3. The sterile medium was dispensed in petri-plates, inoculated with 0.1 ml soil suspension and incubated at 28 ± 2 °C. The colonies showing a zone of proteolysis after 5 days of incubation were isolated and purified. Out of five soil isolates, two were identified as *Aspergillus parasiticus*, one as *Aspergillus flavus*, one as *Penicillium* sp. and one as *Beauveria felina*. All isolates were routinely maintained on PDA slants at 4 °C.

The known protease producing strains *A. oryzae* NCIM 649, *A. oryzae* NCIM 1212 and *A. oryzae* NCIM 1032 were procured from the National Collection of Industrial Microorganisms, Pune, India. *Metarhizium anisopliae* MTCC 892 was purchased from Microbial Type Culture Collection and GeneBank, Chandigarh, India.

2.3. Inoculum preparation

The fungi were grown on modified Czapeck-Dox Agar at 30 ± 2 °C. After profound sporulation, the plates were scrapped with 10 ml of sterile water containing 0.1% Tween-80 under aseptic condition. The spore suspension adjusted to desired count using haemocytometer served as an inoculum.

The entire study was broadly divided into two steps. The first step involved the assessment of proteolytic activity of soil isolates and known protease producing strains of fungi under similar conditions of growth and assay over different incubation periods. The hyper-proteolytic strains from step one were selected for the optimization of parameters for maximum protease production under SSF condition for soy protein hydrolysis.

2.4. Initial screening studies

2.4.1. Growth conditions

One gram of wheat bran supplemented with 0.1% soy protein isolate as an inducer was moistened with 0.8 ml tap water. The tubes were autoclaved at 121 °C for 45 min. After cooling to room temperature, the bran was inoculated with 0.2 ml of spore suspension ($\sim 10^{10}$ spores ml^{-1}). All fungi were incubated in a humidity-controlled incubator (RH = 95%) at 36 °C except *Beauveria felina* and *Metarhizium anisopliae* MTCC 892, which were incubated at 28 °C. The tubes were withdrawn in triplicate on alternate days for extraction of enzyme.

2.4.2. Extraction and assay of protease

The mouldy bran was extracted with 10 ml of NaCl solution (1%), vortexing the suspension intermittently for 1 h. The suspension was filtered under vacuum and the filtrate was used as a crude enzyme extract (CEE). Protease activity was assayed in a reaction mixture containing 1 ml suitably diluted enzyme extract, 5 ml of 2% soy protein isolate dissolved in 0.5 M carbonate buffer (pH 10) and 4 ml carbonate buffer (pH 10; 0.5 M). The reaction mixture was incubated at 45 °C on a gyratory incubator shaker (300 rpm) for 30 min. Aliquot of the reaction mixture (0.5 ml) was withdrawn and the reaction was terminated by addition of 1.5 ml pre-chilled trichloroacetic acid (10%). Complete precipitation of protein was achieved by immersing the tubes in ice bath. The supernatant obtained after centrifugation (7000 rpm, 10 min) was analyzed for tyrosine liberated during hydrolysis following Lowry's method [14]. Unit enzyme activity was defined as the amount of enzyme that liberated 1 μg tyrosine per minute under assay conditions and reported in terms of protease activity per gram initial dry substrate (IDS).

2.5. Optimization of parameters

Based on the results of initial screening, *Penicillium* sp., *Beauveria felina* and *Aspergillus oryzae* NCIM 649 were selected for parameter optimization. The present study discusses the process optimization of *Beauveria felina* and *Aspergillus oryzae* NCIM 649, whereas *Penicillium* sp. has been described earlier [12]. The sequence of parameters optimized during growth was: moistening medium, initial moisture content, incubation temperature, enzyme extractants, inducer concentration, spore concentration and initial pH of the medium. The moistening media chosen for the present study included: M-9, M-15, Czapeck-Dox [5], MS [15] and tap water (TW) [16]. The parameters optimized earlier were incorporated during the subsequent experiments. The effects of various metal salts, chelators and inhibitors on alkaline protease activity were studied during the present study. These reagents were pre-incubated with the enzyme extracts of the chosen fungi at their respective temperature optima for 30 min, with final concentration adjusted to 1 mM in the reaction medium. For ethanol soluble reagents, appropriate control containing enzyme extract-ethanol (1:1, v/v) was incubated.

3. Results and discussion

3.1. Initial screening studies

Protease activity of known protease producer fungal strains and soil isolates grown under SSF condition is presented in Table 1. Although both the strains of *Aspergillus parasiticus* could produce high titres of proteases in a relatively shorter incubation period, they were eliminated from the study due to the detection of aflatoxin in the crude enzyme extracts. However, comparable activity was observed with *Beauveria felina* on day 7. Similarly, comparable results were obtained with *Aspergillus oryzae* NCIM 649 and *Metarhizium anisopliae* MTCC 892 on days 3 and 7, respectively. This was the basis for process optimization of alkaline protease production by *B. felina* under SSF condition and its critical comparison with known proteolytic strain, *A. oryzae* NCIM 649.

Penicillium sp. showed an added advantage to be chosen, as it showed a highest activity on day 3 as compared to *Metarhizium anisopliae* MTCC 892.

Further, various growth parameters were studied under optimum assay conditions for the enzyme extracts of *B. felina* (temperature 55 °C; pH 9 in 0.1 M borate buffer) and *A. oryzae* NCIM 649 (temperature 45 °C; pH 10 in 0.1 M borax NaOH buffer).

Table 1
Protease activity of some known protease producer fungal strains and soil isolates grown under SSF condition after different incubation period

Organism	Incubation period (days)	Protease activity ($U\,g^{-1}$ IDS)
A. oryzae NCIM 649	3	6301 ± 773
	5	2325 ± 180
	7	2231 ± 652
	9	581 ± 172
A. oryzae NCIM 1212	3	1069 ± 149
	5	1238 ± 351
	7	1631 ± 318
	9	1238 ± 203
A. oryzae NCIM 1032	3	1200 ± 234
	5	1631 ± 245
	7	4744 ± 117
	9	487 ± 141
M. anisopliae MTCC 892[a]	3	NG
	5	4350 ± 676
	7	6452 ± 304
	9	3690 ± 664
Beauveria felina (isolate 1)[a]	3	1052 ± 152
	5	4838 ± 298
	7	8211 ± 130
	9	3887 ± 369
A. parasitcus (isolate 2)	3	9545 ± 277
	5	7520 ± 776
	7	5963 ± 398
	9	4632 ± 859
A. parasiticus (isolate 3)	3	9451 ± 718
	5	6938 ± 197
	7	4388 ± 159
	9	3525 ± 844
A. flavus (isolate 4)	3	1894 ± 422
	5	1519 ± 258
	7	1313 ± 234
	9	1041 ± 119
Penicillium sp. (isolate 5)	3	4819 ± 601
	5	3719 ± 145
	7	2897 ± 265
	9	2044 ± 234

Assay temperature 45 °C, pH 10 (carbonate buffer 0.5 M), and growth temperature 36 °C. NG, no growth; data are the mean of three replicates ± standard deviation.

[a] Incubation temperature 28 °C.

3.2. Optimization of parameters

Almost a 20% increase in the enzyme activity was observed in the case of *B. felina*, when tap water was replaced with M-9 solution. Similar trend was observed with *A. oryzae* NCIM 649, where M-15 solution increased the alkaline protease activity by 16% (Fig. 1). More than a two-fold increase in the protease activity of *Rhizopus oryzae* NRRL 21498 has been reported using M-9 solution as moistening medium by Tunga et al. [5]. However, a marginal increase in the enzyme activity with various moistening media indicated that wheat bran moistened with tap water could fulfill all the requirements for growth and protease production by both the fungi.

The requirement of 120% initial moisture content (Table 2) by *Beauveria felina* is agreeable with earlier studies [5], which reported 140% optimum initial moisture content for *Rhizopus oryzae* for alkaline protease production. However, the requirement of moisture content for maximum protease activity differed in *A. oryzae* NCIM 649, which exhibited maximum protease activity at 60% moisture initially added in the bran. *A. niger* and *A. flavus* have also been shown to secrete maximum acid and alkaline proteases, respectively, after 2–3 days of incubation at 52–72% moisture level [17,18], which supports the present findings with *A. oryzae* NCIM 649.

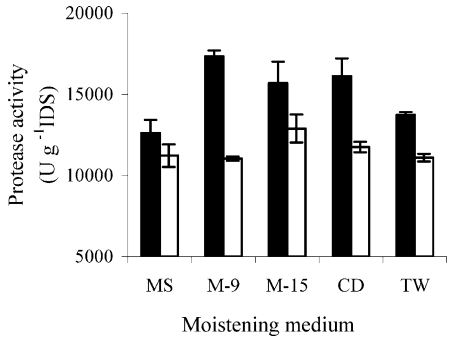

Fig. 1. Effect of moistening medium on the protease activity of *B. felina* (■) and *A. oryzae* NCIM 649 (□) grown under SSF conditions.

Table 2
Effect of initial moisture content on protease activity of *B. felina* and *A. oryzae* NCIM 649 grown under SSF condition

Moisture content (ml g^{-1} IDS)	Protease activity (U g^{-1} IDS)	
	B. felina	*A. oryzae* NCIM 649
0.6	12583 ± 320	10916 ± 162
0.8	15640 ± 168	9325 ± 125
1.0	17172 ± 226	8783 ± 156
1.2	18423 ± 126	8168 ± 322
1.4	16578 ± 320	7075 ± 965

Data are mean of three replications ± standard deviation.

Beauveria felina showed a sharp decline in protease activity, when its incubation temperature was increased from 28 to 36 °C (Fig. 2). Most of the fungi showed an optimum temperature range between 28 and 30 °C for protease production under SSF condition as described earlier [17–19]. However, *A. oryzae* NCIM 649 seems to tolerate higher temperature, attaining maximum protease activity at 36 °C. A combination of higher incubation temperature and local heat generated during solid substrate fermentation could be the reasons for severe adverse effect on protease production in the case of *B. felina*.

Of various extractants employed, 1% NaCl solution proved to be most suitable for maximum recovery of alkaline protease from both *B. felina* and *A. oryzae* NCIM 649 (Table 3). Adsorption of the enzyme to mouldy bran has been attributed to ionic bond, hydrogen bond and Van der Waal's forces [20]. Sodium chloride (1%) has been shown to be the most suitable protease extractant for *Mucor bacilliformis* [20] and *Rhizopus oligosporous* [21]. Alternatively, an aqueous mixture of ethanol (10%) and glycerol (3%) has been efficiently used for the extraction of *Rhizopus oryzae* protease [22]. The chemical composition of fungal mycelia appears to determine the suitability of protease extractant.

Although wheat bran contains protein, supplementing it with soy protein isolate as an inducer lead to an interesting observation. Almost 12-fold increase in the protease activity was observed when *A. oryzae* NCIM 649 was grown on soy

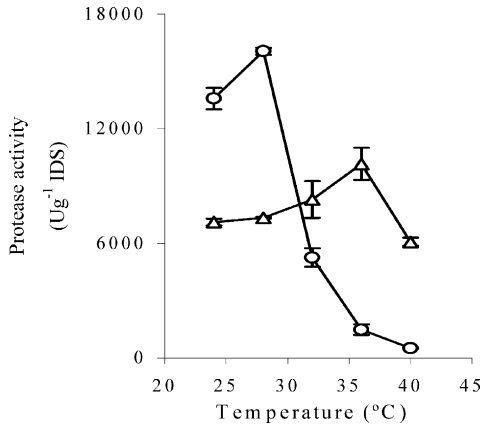

Fig. 2. Effect of incubation temperature on the protease activity of *B. felina* (○) and *A. oryzae* NCIM 649 (△) grown under SSF conditions.

Table 3
Effect of enzyme extractants on protease activity of *B. felina* and *A. oryzae* NCIM 649 grown under SSF condition

Extractant	Protease activity (U g^{-1} IDS)	
	B. felina	*A. oryzae* NCIM 649
NaCl (1%)	23319 ± 1487	12043 ± 263
Ethanol (10%) + glycerol (3%)	21010 ± 1268	10372 ± 957
Tween-80 (0.5%)	20609 ± 429	10083 ± 756
Distilled water	20757 ± 966	10194 ± 672
Buffer[a]	21828 ± 705	10119 ± 273

Data are mean of three replications ± standard deviation.
[a] Represent the optimized buffer in which the activity of both the fungi was maximum.

protein supplemented wheat bran (15 mg g^{-1} IDS). Increase in protease activity of *B. felina* was also observed by the addition of soy protein in wheat bran, but to a lesser extent (Fig. 3). Conflicting reports are available on protease activity of fungi grown on protein supplemented wheat bran. *Aspergillus niger var. tieghem* showed about 1.4-fold increased acid protease activity in presence of casein and traders' protein added in wheat bran [17]. On the contrary, 6, 14 and 55% reduction in alkaline protease activity of *Aspergillus flavus* IMI 327634 was recorded by supplementing wheat bran with casein, traders' protein and gelatin, respectively [18]. Induction of protease activity by supplementing wheat bran with protein appears to be dependent on the fungal strain used for the experiment.

An exponential increase in the enzyme activity of both fungi was recorded with increasing concentration of spores in the inoculum (Table 4). For *Rhizopus oryzae* NRRL 21498, the optimum spore concentration required for maximum protease activity was $\sim 2 \times 10^5$ spores g^{-1} wheat bran and further increase in the size of inoculum did not reveal increased protease activity [5]. However, in the case of both fungi used in the present study, no such optimum inoculum size could be determined up to a very high spore count of 10 billion spores g^{-1} wheat bran.

The studies of initial pH suggested that both the fungi produced protease optimally at pH 7 (Fig. 4). A sharp

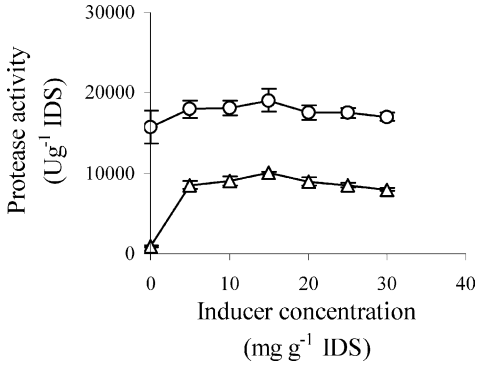

Fig. 3. Effect of inducer concentration on the activity of *B. felina* (○) and *A. oryzae* NCIM 649 (△) grown under SSF condition.

Table 4
Effect of spore concentration in the inoculum on protease activity of *B. felina* and *A. oryzae* NCIM 649 grown under SSF condition

Logarithm of spore concentration (g IDS^{-1})	Protease activity (U g^{-1} IDS)	
	B. felina	*A. oryzae* NCIM 649
5	7501 ± 277	9365 ± 90
6	9132 ± 510	10047 ± 566
7	12226 ± 32	10439 ± 380
8	14158 ± 212	10882 ± 150
9	14664 ± 534	11514 ± 324
10	19747 ± 554	12093 ± 515

Data are mean of three replications ± standard deviation.

decline (99.2%) in the activity of *B. felina* was observed when initial pH of the bran was adjusted to 4, whereas about 78% reduction was recorded with *A. oryzae* NCIM 649. The differential sensitivity of protease producing fungal strains towards pH has also been pointed earlier [5].

Pre-incubation of culture filtrates with various metal salts revealed prominent inhibitory effect of Fe^{3+}, Hg^{2+} and Cu^{2+} ions on alkaline proteases of both the fungi (Table 5). Severe enzyme poisoning with Zn^{2+} in *Beauveria felina* was recorded as also observed earlier with *Penicillium* sp. LPB-5 [19]. Protease extracted from *Trichoderma koningii* revealed similar inhibition in the presence of Hg^{2+} and Fe^{3+} [4]. The inhibition of bacterial proteases by Hg^{2+} and Zn^{2+} ions has also been repeatedly shown [23,24]. The presence of Ca^{2+}, Mg^{2+} and Mn^{2+} ions not only protected the enzyme from undergoing denaturation but also enhanced the activity marginally. Thermal stability imparted to proteases by these salts reconcile with earlier observations [2,25]. Inhibition of 90 and 74% protease activity of *Beauveria felina* and *A. oryzae* NCIM 649, respectively, by PMSF indicates that both are alkaline serine proteases (Table 6). Cupferron, 1,10-phenanthroline, EGTA and EDTA increased the protease activity of *Beauveria felina* marginally indicating efficient chelation of inhibitory metal ions, which may be present in trace amounts. Severe inhibition was observed

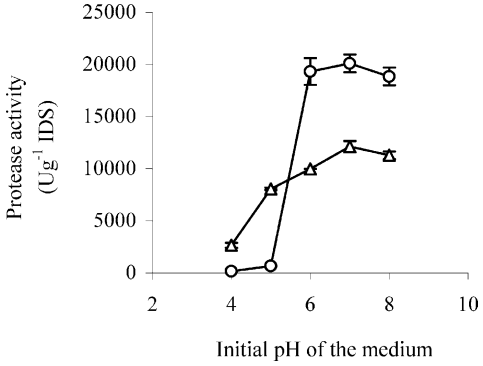

Fig. 4. Effect of initial pH of the medium on the activity of *B. felina* (○) and *A. oryzae* NCIM 649 (△) grown under SSF condition.

Table 5
Effect of metal salts on protease activity[a] of culture filtrates of *B. felina* and *A. oryzae* NCIM 649 grown under SSF condition

Metal salts (1 mM)	Residual protease activity (%)	
	B. felina	*A. oryzae* NCIM 649
Control	100.0	100.0
$FeSO_4$	83.5	26.5
$FeCl_3$	6.35	6.3
$ZnSO_4$	1.47	70.9
$HgCl_2$	3.4	8.4
$CoCl_2$	94	65.0
$(CH_3COO)_2Pb$	89.03	59.7
$CaCl_2$	115.7	99.0
$MgSO_4$	108.9	124.2
$MnSO_4$	106.9	108
$CuSO_4$	2.47	7.4

[a] The activity is expressed as a percentage of the activity in absence of metal salts. The enzyme extracts were pre-incubated with the metal salts for 30 min at respective assay temperatures.

with sodium dodecyl sulphate (SDS) in case of *B. felina* but *A. oryzae* NCIM 649 was able to retain about 87% activity. This result showed the possibility that the protease of *B. felina* might be a multimeric protein with quaternary structure. The enzyme extract of *B. felina* showed resistance to denaturation in ethanolic solution. In contrast, 71% reduction in protease activity of *A. oryzae* NCIM 649 was recorded under similar conditions. It also exhibited prominent inhibition with αα′-dipyridyl and 1,10-phenanthroline.

The present work indicated the potentiality of *Beauveria felina* as a new source of alkaline protease, grown under SSF conditions. The production of soy protein hydrolysate using alkaline protease by *B. felina* and its characterization is in progress.

Table 6
Effect of metal chelators and inhibitors on protease activity of *B. felina* and *A. oryzae* NCIM 649 grown under SSF condition

Chelators/inhibitors (1 mM)	Protease activity (U g^{-1} IDS)	
	B. felina	*A. oryzae* NCIM 649
Control (CEE:distilled water, 1:1)	14777 ± 253	11664 ± 425
Urea	13314 ± 64	11540 ± 739
SDS	1031 ± 32	10164 ± 749
EDTA	14796 ± 737	11870 ± 446
EGTA	15358 ± 1181	11683 ± 1081
Cupferron	16596 ± 148	14702 ± 212
Control (CEE:ethanol, 1:1)	16521 ± 395	3375 ± 202
αα′-Dipyridyl[a]	16446 ± 180	468 ± 32
8-Hydroxyquinoline[a]	13577 ± 437	2363 ± 97
PMSF[a]	1243 ± 87	881 ± 32
1,10-Phenanthroline[a]	16896 ± 117	562 ± 56

Data are mean of three replications ± standard deviation. The chelators/inhibitors were pre-incubated with the enzyme extracts for 30 min at respective assay temperatures.

[a] The chelators/inhibitors were dissolved in ethanol.

Acknowledgements

Financial assistance to one of the authors (T.B.) by Council of Scientific and Industrial Research, New Delhi, is thankfully acknowledged.

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