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RESEARCH ARTICLE

Optimization and production of extracellular alkaline protease by solid state fermentation using *Bacillus subtilis*

G. Prabhavathy[1], M. Rajasekara Pandian[2] and B. Senthilkumar[3]
[1]Dept. of Biotechnology, Selvam Arts and Science College, Namakkal, TN, India
[2]Dept. of Zoology, Arignar Anna Government Arts College, Namakkal, TN, India
[3]Dept. of Medical Microbiology, Haramaya University, P.O. Box 235, Harar, Ethiopia
prabharam1969@gmail.com; rsp_biotech@yahoo.com; +91 9842761861; +251-931907924

Abstract

A total of seven samples of bio-effluent were collected from leather processing company in Tirupur district, TN and the isolated bacterial strains from the effluent (B1 to B7) were screened for extracellular alkaline protease production. Bacterial isolates (B1, B3, B4, B5 and B6) inoculated on skim milk agar medium produced clear zone around the colony indicating protease activity. Among them, isolate B6 produced high activity and was identified as *Bacillus subtilis* by biochemical and molecular characterization. In this study, cultural environment for protease production was optimized using solid state fermentation (SSF). The results revealed that wheat bran, glucose, peptone, pH 7.5, temperature of 45°C and incubation time of 96 h enhanced protease production.

Keywords: Bio-effluent, leather processing, alkaline protease, skim milk agar, *Bacillus subtilis,* wheat bran.

Introduction

Proteases are enzymes that hydrolyze proteins via the addition of water across peptide bonds and catalyze peptide synthesis in organic solvents and in solvents with low water content (Sookkheo *et al*., 2000). Proteolytic enzymes are ubiquitous in occurrence found in all living organisms and are essential for cell growth and differentiation. There is renewed interest in the study of proteolytic enzymes mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial community (Gupta *et al*., 2002). Proteases are produced commercially and used in detergent formulation, leather and dairy industries (Anwar and Saleemuddin, 1998; Dias *et al*., 2008). Proteases have a long history of application in the food and detergent industries. Their application in the leather industry for dehairing and bating of hides to substitute currently used toxic chemicals is a relatively new development and has conferred added biotechnological importance of protease. Substrates used in the production medium for protease production is very high and a new feasible technology employing cheap available substrates are needed at this point and in recent years solid state fermentation has gained importance owing to certain advantages over the conventional submerged fermentation.

In view of the above facts, this study was aimed to optimize the cultural environment for protease production by *Bacillus subtilis* isolated from effluent of leather processing company in Tirupur, TN. Effect of agro-wastes, carbon and nitrogen sources, pH, temperature and incubation time on alkaline protease production was optimized using solid state fermentation.

Materials and methods

Collection and processing of effluent: The effluent was collected from a storage reservoir and outlet pipes from the discharge point of tannery unit of leather processing company in Tirupur, TN. The effluent was sampled in dry sterile, polypropylene bottles which were kept in ice during transportation and stored in the refrigerator (4°C) until further use.

Isolation of bacteria: Bacterial population of effluent was isolated by serial dilution and plating method. The sample was diluted from 10^{-4} to 10^{-7} and 0.1 mL of the resulting liquid from each dilution was spread on the surface of Nutrient agar medium using an L-shaped glass rod and incubated at 37°C for 24 h. The bacterial isolates formed were sub-cultured in the same Nutrient agar medium (Hi Media, India) to purity.

Screening of bacterial isolates for protease production: Microorganisms isolated from the effluent were plated on skim milk agar plates containing peptone (0.1%), NaCl (0.5%), agar (2%) and skim milk (10%), incubated at 37°C for 48 h. A clear zone of skim milk hydrolysis appeared after 48 h incubation and depending upon the zone of clearance, the potential strain was selected. The selected potential isolates for production of protease was sub-cultured and maintained on agar slants. The slants were stored at 4°C for further analysis.

Identification of bacteria: Bacterial identification was carried out by morphological and biochemical characterizations namely gram Staining, motility test, catalase, oxidase, carbohydrate fermentation test, indole, methyl red, citrate utilization test and Voges Proskauer test (Senthilkumar *et al.,* 2012).

The strain was inoculated on nutrient and blood agar and incubated at 37°C for 24 h and the colony morphology was identified.

Optimization of culture conditions for alkaline protease production: Agro-industrial waste materials namely wheat bran, soy cake, coconut cake and groundnut cake was selected as substrates for protease production by solid state fermentation in the production media (Glucose 1.5 g%, Urea 2 g%, KH_2PO_4 0.2 g%, $MgSO_4.7H_2O$ 0.1 g%, $CaCl_2$ 0.1 g%, substrates 7.5% (w/v). For this, 10 g of substrate is taken in separate flasks and autoclaved at 121°C and 15 lb pressure for 20 min. After cooling, the flasks were inoculated with equal quantity of inoculums. After 24 h of incubation, the enzyme was extracted using 0.2 M phosphate buffer (pH 7.2) and enzyme activity was assayed according to Mukhtar and Ikram-ul-haq (2008). The best substrate which secreted high protease activity was selected for further process. Carbon sources namely glucose, sucrose, starch, fructose, maltose and cellulose were amended separately in flasks inoculated with equal quantity of inoculums. Organic and inorganic nitrogen sources namely ammonium sulphate ($(NH_4)_2SO_4$), ammonium chloride (NH_4Cl_2), ammonium nitrate ($NaNO_2$), urea and peptone were amended in the production media separately. The same extraction procedure was followed and protease activity was assayed.

To observe the effect of initial pH on enzyme production, 10 g of production media of different pHs (4, 5, 6, 7, 7.5 and 8) was taken in each flask. Similarly, the culture media was incubated to find out the effect of different temperatures (27, 37, 47, 57 and 67°C) on protease production. The effect of incubation periods on the protease activity by the test isolate was studied. For protease production, culture media was incubated at different time intervals namely 24, 48, 72, 96 and 120 h.

Results and discussion

A total of seven samples of bio-effluent were collected for screening the bacterial isolates (B1 to B7). They were inoculated on skim milk agar medium. After incubation period, skim milk agar revealed that the isolates were capable of producing clear zone around the colony growth indicating positive protease producers. Of the samples collected, five isolates (B1, B3, B4, B5 and B6) showed proteolytic activity, whereas two bacterial isolates were negative for proteolytic activity (Table1; Fig. 1). Among the proteolytic isolates the one (B6) which showed highest zone in the skim milk agar was used for the further studies. This isolate was identified as *Bacillus subtilis* based on the colony morphology which was large, irregular and flat with an undulate margin on nutrient agar (Fig. 2) and biochemical characteristics (Table 2).

Table 1. Screening of protease producing bacterial isolates.

Isolates	Zone of inhibition (mm)
B1	10
B2	-
B3	11
B4	9
B5	6
B6	14
B7	-

Fig. 1. Screening of proteolytic bacteria on skim milk agar.

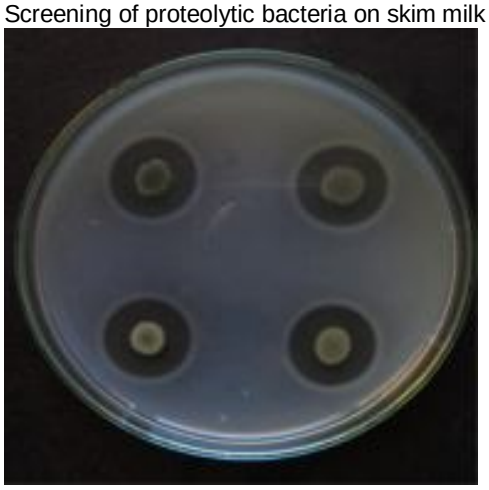

Fig. 2. *Bacillus subtilis* on nutrient agar.

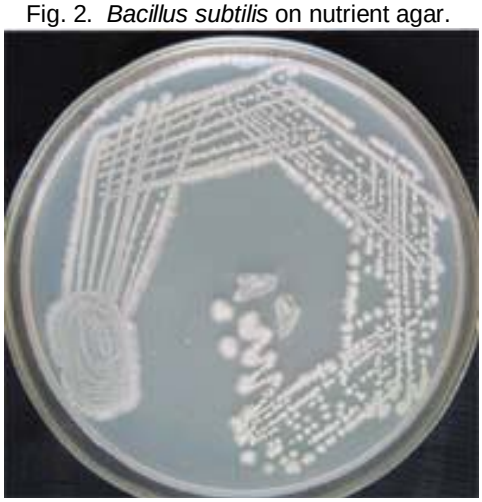

Table 2. Biochemical tests for *Bacillus subtilis.*

Biochemical tests	Results
Catalase test	+
Oxidase test	Differentiate
Urease test	-
Indole test	-
Methyl red test	-
Voges Proskauer test	+
Citrate utilization test	+

The isolated *Bacillus subtilis* showed β-haemolytic colonies on blood agar. The selected colonies of *Bacillus subtilis* was cultured separately on skim milk agar which showed clear zone capable of producing protease around the colony. The test isolate when cultured in the starch agar proving that it is capable of utilizing starch and this one of the important characteristic of *Bacillus* sp. Further confirmation of the test isolate was done by sequencing the 16S-rDNA gene and compared with the GenBank databases using the BLASTN program (data not shown). Therefore, all these biochemical and cultural characteristics and molecular characterization proved that representative protease producing *Bacillus subtilis.*

Alkaline proteases are produced by a wide range of microorganisms including bacteria, molds, yeasts and also mammalian tissues. Of all the alkalophilic microorganisms used in various industrial applications, members of the genus *Bacillus* were found to be predominant and a prolific source of alkaline protease (Deng *et al*., 2010). Soy cake, coconut cake, wheat bran and groundnut cake were used for the production of protease by SSF. Among these substrates wheat bran showed high protease activity (Table 3). Many studies used the natural sources as one of the medium constituents such as rice bran, soybean, wheat flour, what bran, corn bran, corn starch and orange peels to support growth of different bacteria to produce protease (Joo and Chang, 2005). Glucose was found to be the best carbon source whereas an organic nitrogen source peptone favored protease production (Table 4 and 5). There is an increasing interest in the last few years based on working on molasses due to the possibility to use it as a cheap carbon and energy sources not only to cultivate microorganisms on it, but also to obtain valuable products, which have different applications with economic importance relatively inexpensive and economic alternative to synthetic medium for the production of some bi-product (El-Enshasy *et al.,* 2008).

Atalo and Gashe (1993) showed that yeast extract and peptone can induce the alkaline protease production. Although yeast extract and peptone are rich in protein and amino acid components, didn't induce production of alkaline protease in *B. polymixa* and *B. cereus*. pH and temperature are the two important environmental factors which affect the protease production of any bacteria. The pH of the production medium play a critical role for the optimal physiological performances of the bacterial cell and the transport of various nutrient components across the cell membrane aiming at maximizing the enzyme yields. A neutral pH was found to be optimum for protease production where as 45°C supported the same (Table 6 and 7). Kobayashi *et al.* (1996) reported that optimal activity of bacterial protease at pH 11 and Kumar (2002) reported that the production of an alkaline protease at pH 11.5 from *Bacillus* sp. High protease production was recorded after an incubation time of 96 h by *Bacillus subtilis* (Table 8).

Table 3. Effect of substrates on protease production.

Substrates	Enzyme activity (U/g)
Soy cake	344.12 ± 0.84
Coconut cake	444.12 ± 1.12
Wheat bran	496.12 ± 1.65
Groundnut cake	414.12 ± 1.42

Table 4. Effect of carbon source on protease production.

Carbon source	Enzyme activity (U/g)
Glucose	490.24 ± 1.29
Fructose	240.12 ± 2.40
Sucrose	162.12 ± 1.22
Maltose	300.14 ± 0.46
Cellulose	122.12 ± 2.12
Starch	214.12 ± 2.03

Table 5. Effect of nitrogen source on protease production.

Nitrogen source	Enzyme activity (U/g)
$(NH_4)_2SO_4$ (10 mM)	2873.9 ± 355.9
NH_4Cl_2 (10 mM)	1669.9 ± 67.3
$NaNO_2$ (10 mM)	1825.3 ± 67.2
Urea (1%)	2019.5 ± 177.9
Peptone (1%)	3184.6 ± 269.1

Table 6. Effect of pH on protease production.

pH	Enzyme activity (U/g)
4	1575.8 ± 62.1
5	1626.5 ± 124.5
6	1693.3 ± 159.1
7	1851.8 ± 167.9
7.5	1771.0 ± 120.1
8	1640.4 ± 62.3

Table 7. Effect of temperature on protease production.

Temperature (°C)	Enzyme activity (U/g)
27	361.1 ± 92.4
37	326.2 ± 38.8
45	442.7 ± 51.9
55	404.2 ± 54.1
65	311.1 ± 12.0

Table 8. Effect of incubation time on protease production.

Incubation time (h)	Enzyme activity (U/g)
24	1220.4 ± 53.3
48	1444.8 ± 11.8
72	1666.5 ± 57.1
96	1803.0 ± 127.5
120	1725.4 ± 72.5

Gradual decreasing enzyme unit was observed with increasing inhibition period clearly suggesting the enzyme's role as a primary metabolite being produced in the log phase of growth of the bacteria for utilization of nutrients present in the solid substrate.

Conclusion

Bacterial strains from the effluent (B1 to B7) were screened for extracellular alkaline protease production. Bacterial isolates (B1, B3, B4, B5 and B6) inoculated on skim milk agar medium produced clear zone around the colony indicating protease activity. Isolate B6 produced high activity and was identified as *Bacillus subtilis* by biochemical and molecular characterization. Cultural environment for protease production was optimized using solid state fermentation ant it was found that wheat bran, glucose, peptone, pH 7.5, temperature of 45°C and incubation time of 96 h enhanced protease production. Hence this little piece of investigation may be used for commercial production of economically valuable proteases by utilizing agro-industrial wastes in near future.

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