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ISOLATION AND CHARACTERIZATION OF THERMOSTABLE PROTEASE PRODUCING BACTERIA FROM TANNERY INDUSTRY EFFLUENT

T. R Srinivasan*, Soumen Das, V. Balakrishnan, R. Philip, N. Kannan
Department of Biotechnology, K.S.Rangasamy College of Technology, Tiruchengode, Tamil Nadu - 637 215, India

Abstract

The study is a search for potential thermostable protease producing strains from tannery industry effluent. Among nine protease producing strains screened, one was selected as promising thermostable protease producer and identified as *Bacillus* sp. The activity of the protease produced by this organism is stable up to 70^0C. The optimum yield was achieved after 48 hours of culture, at 60^0C with the pH 8.0. The desired protein was precipitated from the crude extract by using ammonium sulfate (70%) followed by dialysis and purified by Ion-exchange chromatography. The maximum protease activity was observed at 65^0C and at pH 8.0. Further investigation on structure elucidation to this purified protein for industrial level exploitation is in progress.

Key Words: Thermostable protease; *Bacillus* species; Tannery industry effluent.

Introduction

The industrial enzyme represents a major part of the global enzyme business and it has grown about $ 1.4 US billion annually. The scope of Industrial enzymes is growing because they i) offer less polluting processes than chemical catalyst ii) perform reactions with higher specificity than chemical catalyst and iii) perform reaction for which chemical catalyst are not known [1]. Among these enzymes, protease is the most important enzyme and accounts for about 60% of the total worldwide sale [2]. With respect of properties of this enzyme and its capacity for degradation of different protein source, these proteases having a long history of application in different industries viz. detergents, food-brewing [3,4], meat tenderization, baking, manufacture of soya products, diary, leather [5,6]. Mostly, all industrial processes are carried out at higher temperature where normal enzymes become unstable [7,8]. Therefore there is an ample scope for searching thermostable proteases which can resist the changes in environmental conditions in which they are supposed to work in industries. A number of bacteria have been newly isolated from the hot springs over past few years [9,10]; however information on extra-cellular proteolytic enzyme producing bacteria from non-thermal source has been scarce so far. In pursuit for the search of potential thermostable protease producing bacteria from tannery industry effluent, tannery industry effluent is selected because of its content of natural proteins [11,12,13]. The extra cellular thermostable protease and its features suggest its application in detergent industry which generally use protease synthesized by chemical methods.

Materials and methods

Collections of samples

The protein rich tannery effluent samples were collected from different tannery industries of Tamil Nadu state, India. Effluent samples were stored in a cold chamber at 4^0C for future use. Temperature, pH, BOD and COD data of each sample has been collected from the respective industries [14].

Isolation of thermostable protein producing bacteria

Enumeration of total heterotrophic population from sample was performed by dilution agar plating technique.

* *Corresponding Author*

The grown colonies are checked for their protease producing capability by inoculating them in autoclaved skimmed milk medium and incubated at 37^{0}C for 24-48 h. Depending on the diameter of the clear zone around the colonies, nine protease producing bacteria were selected and were maintained by streaking them onto agar slant and storing them at 4^{0}C in the refrigerator. Further, they were checked for the thermal stability. All the nine cultures was inoculated in nine different Erlenmeyer flasks containing 50mL Nutrient broth each and incubated at 50^{0}C for 36 hours in water bath shaker (Narang Scientific Works Pvt. Ltd., New Delhi). After the incubation period 1ml of broth was plated in casein-agar plate and the remaining broth was incubated at 60^{0}C. This method was followed up to 80^{0}C. Plates were observed for getting the thermostable protease producing bacteria.

Identification of the isolated bacteria

Gram staining, spore staining, negative staining and biochemical tests were done to identify the isolated bacteria. The following tests were done for this purpose. Methyl red test, Voges-Proskaur test, Triple Sugar Iron test, Indole test, Catalase test, Nitrate reduction test, Hydrolysis of starch, citrate utilization test.

Optimization of the growth conditions of the selected bacteria: Batch fermentation

Batch fermentation was carried out in 250mL Erlenmeyer flaks containing 50mL of Nutrient broth media. Fermentation was initiated by inoculating 1% $^V/_V$ of starter culture incubated at 30^{0}C for overnight period in shaking incubator (Labline Instruments, Cochin).

Effect of Carbon and Nitrogen sources on protease production

In the successive experiments, either the carbon source (0.2% $^W/_V$) or the nitrogen source (1% $^W/_V$) was added to Nutrient broth. Galactose, glucose, sucrose, lactose, fructose, manitol were used as carbon sources. Typtone, peptone, beaf-extract and glutamic acid were used as nitrogen sources.

Determination of the protease activity

Protease activity was determined and the method was adopted from Bhosale et al.(5) using casein as the substrate. Enzyme solution was added to 3.0 mL of substrate solution and the mixture was incubated at 60^{0}C for 20 min. The reaction was stopped by addition of 3.2mL of TCA mixture and kept at 60^{0}C for 30 minute followed by filtration. The absorbance of the filtrate was measured by spectrophotometer (Genesys 100V: Thermos Spectronic, USA) at 280nm. One unit of protease activity is defined as the amount of enzyme required to produce 1µg of tyrosine per minute under the condition described. Each data point is the average of at least three determinations.

Purification of the thermostable protease

The thermostable protease from the isolated bacteria was partially purified by performing the ammonium sulfate precipitation, dialysis and ion-exchange chromatography. The molecular weight was determined by performing the SDS-PAGE (12% resolving gel) and broad range marker (Medox, India) was used.

Optimization of enzyme activity

The effect of pH and temperature on the rate of reaction was studied by assaying elute in the range of pH 4.0 to 10.0and in the following temperature: 4^{0}C, 30^{0}C, 50^{0}C and 70^{0}C.

Results and discussion

A new thermostable protease producing bacteria was isolated from tannery industry effluent and the stability was checked (Table 1).The bacteria identified and biochemical results given in the table 2. It showed excellent growth at 60^{0}C on nutrient medium. The widest zone diameter (1.2 cm) was obtained for this organism among the others screened and showed protease activity of 2.21 IU/mL/min.

Table 1. Stability of the thermostable protease produced by the selected bacteria

Temperature (^{0}C)	O.D at 280 nm	Protease activity (IU/mL/min)
50	0.985	1.44
55	1.125	1.64
60	1.50	2.19
65	1.524	2.23
70	1.510	2.21
75	0.248	0.36

The protease produced by this organism was found to withstand up to 70^{0}C. This organism was selected as the thermostable protease producing bacteria. The growth profile showed that its lag phase lasted for 20 hrs. After the lag phase protease started and the maximum protease production was observed in its late log phase, i.e., after 48 h. Preliminary experiments showed that the strain was Rod shaped, spore forming, gram positive bacteria. It showed positive reaction for catalase, MR, VP, hydrolysis of starch, decomposition of citrate and negative reactions for urease, indole, growth on Mc.Conkey agar and nitrate reduction. The morphology

and the biochemical data on the growth of the bacteria indicated that the bacteria belong to *Bacillus* species. However, molecular study and ribotyping method will reveal the true identity of the bacteria. Isolation and staining of bacteria given in Fig. 1.

Table 2. Identification of the isolated bacteria (biochemical tests results)

Biochemical Tests	Bacterial response
Gram's Reaction	+
Shape	Rod
Spore formation	+
M-R	+
V-P	+
Indole	-
Catalase	+
Urease	-
TSI	A/A (Slant/But)
Starch Hydrolysis	+
Acid-fast Stain	-
Nitrate reduction	+/-
Citrate Utilization	+
Casein Decomposition	+
Acid from glucose	+
Growth on Mc. Conkey agar	-

[A=acid production; K=alkaline reaction] [Slant (Acid = yellow | Alkaline=red), Butt (Acid = yellow | Alkaline = red)].

Table 3. Protease activity of different Elutes after Ion-Exchange chromatography

Elute	Protease activity (IU/mL/min)
Elute 1	1.21
Elute 2	1.12
Elute 3	1.43
Elute 4	3.25
Elute 5	1.30

The effect of carbon source on the secretion of extra cellular protease production by the isolated bacteria was investigated under a variety of carbon sources (0.2% $^{W}/_{V}$) such as galactose, glucose, sucrose, lactose, manitol and fructose. Galactose and glucose enhanced the protease secretion by 40% and 35% respectively (Fig. 2). Nitrogen rich medium, containing (1% $^{W}/_{V}$) nitrogen source such as tryptone, peptone, beaf-extract and glutamic acid was used and protease secretion was found to be increased by 46% in case of tryptone while beaf-extract was comparable (Fig. 3).

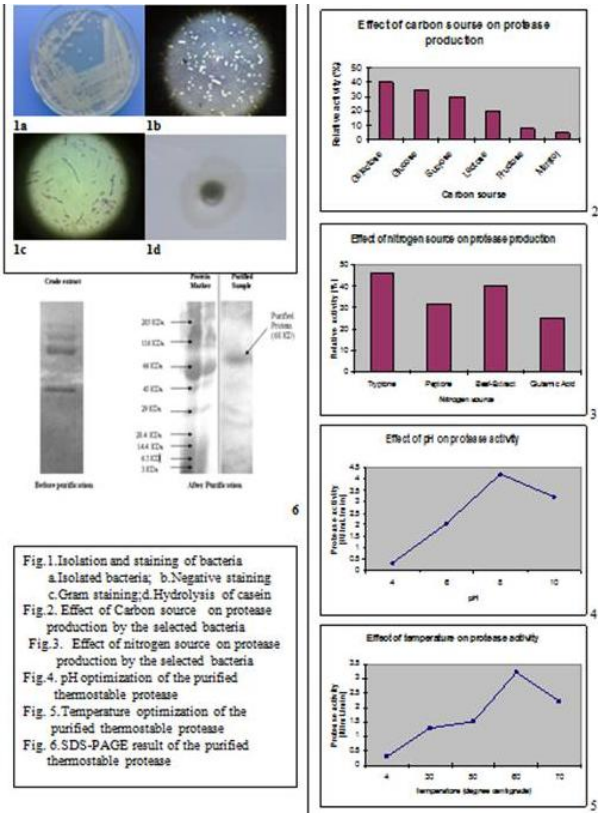

Fig.1.Isolation and staining of bacteria a.Isolated bacteria; b.Negative staining c.Gram staining;d.Hydrolysis of casein
Fig.2. Effect of Carbon source on protease production by the selected bacteria
Fig.3. Effect of nitrogen source on protease production by the selected bacteria
Fig.4. pH optimization of the purified thermostable protease
Fig. 5.Temperature optimization of the purified thermostable protease
Fig. 6.SDS-PAGE result of the purified thermostable protease

Precipitation step was carried out with 50% to 80% ammonium sulfate. However, at 70% saturation most of the protease enzyme was precipitated out. The salt content was removed by performing the dialysis step and the protein was further purified by performing the Ion-exchange chromatography (Table 3). Similar results have been reported [15,16 and 17]. DEAE Cellulose was used as the packing material. The protease activity in the elute was found to be 3.25 IU/mL/min [18]. The quality of the purified protein was also checked by running the elute in the SDS gel (Fig.6) and it was confirmed by observing the single band. By comparing with the molecular marker the molecular weight of the thermostable protease was found to be 68 KDa. The findings validates those observations by Nielsen *et al.* [19] and Wilhelm [20].Though the protease showed activity at wide range of pH 4.0 to 10.0, the optimum activity was found to be at pH of 8.0. The optimum temperature for purified protein activity was 60^{0}C while it can withstand the temperature up to 70^{0}C (Fig 4 and 5).

It thus became imperative to find new methods in order to decrease and/or re-use these wastes. The use of proteases would also make it possible to reduce the use of harmful chemicals and wastes in the environment because these chemicals can be replaced by these

newly produced proteases. Thus, the present work demonstrate the effectiveness of the production of thermostable proteases by thermophilic bacteria using municipal wastewater sludge as a culture medium. This would also reduce the quantity of sludge for final disposal. The quantity of biosolids produced by community wastewater treatment plants can now be reduced by reuse of them. Eventually, all biosolids can be recycled into new eco-friendly products like biopesticides, bioplastics, biocatalysers or in any kind of biogas. It proves that high protease activity can be efficiently extracted from activated sludge. Given the central role extracellular enzymes play in the process of organic matter removal in wastewater treatment processes, and the mildness of the extraction procedure, the enzyme thus extracted can serve as a good starting material for further detailed biochemical and molecular characterization.

Conclusion

It was concluded that the isolated *Bacillus* strain having potential to produce thermostable protease. In general, thermostable protease producing bacteria was found in hot springs or any other thermal source. However, the present study shows the existence of *Bacillus* strain which can produce thermostable industrially important enzyme by existing in the non-thermal source such as tannery effluent. The isolated strain can grow in Nutrient medium. The highest protease activity was achieved at pH 8.0 and temperature at 60^0C. The molecular weight of the purified thermostable protease was found to be 68KDa. Further investigations are needed on genetic analysis of this *Bacillus* strain and structure elucidation of the purified protein for industrial exploitation.

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