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Bacterial Extracellular Alkaline Proteases and its Industrial Applications

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Abstract

Microbial alkaline proteases are among the important hydrolytic enzyme and have been used extensively since the advent of enzymology. Bactrial extracellular alkaline proteases are of great importance due to its wide spectrum applications in detergent industries, bioremediation, food industries, and leather processing and bio-film degradradation. From the various niches eighteen isolates were screened for alkaline protease production, out of which six isolates showed efficient enzyme production. The ratio of zone diameter to colony diameter of six isolates ranged from 2.11 to 7.33. Out of six two isolates B.pumilus p1 and S.auricularis p18 showed significant enzyme activity. Optimization of the incubation period, pH and temperature conditions for enzyme production were determined and were found to be 72hrs, 8.0- 9.0 and 45^{0}C respectively. Optimization of nitrogen sources for enzyme production were determined and was found to be 0.9% for peptone and 0.5% for yeast extract for both the isolates. Enzyme activity was assayed using tyrosine-casein method. The purified enzyme preparations of B.pucilum and S.auricularis having enzyme activity 0.065U/ml and 0.038U/ml respectively were also excellent in dehairing and depilating of raw leather, recovery of silver from X-ray photographic films and bacterial biofilm degradation.

Keywords: Alkaline protease, leather processing, bio-film degradation, *B.pumilus, S.auricularis.*

Introduction

Protease find wide spread applications in food processing, pharmaceuticals, leather processing, bio-film degradation, silver recovery from waste photographic film and various industrial sectors [1]. Extracellular proteases that can be purified easily have been commercially exploited to assist protein degradation in the various industrial processes[2]. Recent awareness of the environmental pollution caused by chemical based industries has necessitated the development of enzyme based processes as alternatives to the currently employed chemical processes[3]. Proteases have potential applications in the industries for partial or total replacement of currently employed toxic chemical processes[4].

Proteases from marine microorganisms are currently receiving increase attention due to their inherent stability at different values of pH, temperature and salinity[1,5,6].

Proteases to be used as detergent additives should be stable and active in presence of detergents[7]. They are used as an active ingredient in the development of biopharmaceutical products like contact lens cleaners and in cleaning the wide variety of polymeric protein substrates[1]. They have extensive applications in different industries viz. food, pharmaceuticals, silk, leather, and recovery of silver from waste X-ray photographic films [2,8].

In the present study of applications of purified alkaline proteases from the *Bacillus* and *Staphylococcus* species, the encouraging results were found in the dehairing and depilating of raw leather[8], recovery of silver from X-ray photographic films[9] and bacterial biofilm degradation[10].

Material and Methods

Sample: soil samples from the vicinity of mutton market, fish market, similarly waste water sludge, sea water, and fermented fish infusion were used to prepare initial inoculums.

Isolation: Isolation of alkaline protease producer was carried out using casein-yeast extract- peptone (CYP) agar medium containing (g/l) Casein-10; Bacteriological peptone-5; Yeast extract-1; K_2HPO_4 -1,$MgSO_4.7H_2O$-0.2, $CaCl_2$-0.1; Na_2CO_3-10; Agar -15. pH- 8-9, all the salts were autoclaved separately and added to the rest of the medium after cooling. The pH of the medium was adjusted using Na_2CO_3 solution[11]. Spread plate techniques was used to inoculate the plates and incubated at 37°C for 48 hours. Based on zone of casein hydrolysis, different isolates were selected and maintained on agar slants.

Screening: A suitably diluted cultures, 0.2ml was spread on CYP agar plate or nutrient agar plate with 1% casein was incubated at 37^{0}C for 48 hrs. Thees enriched samples were plated over nutrient agar containing 0.4% gelatine [12, 13, 14]. After incubation for 24 hrs plates were flooded with 1% tannic acid solution. Colonies showing clear zone of gelatine hydrolysis were picked and purified. Also isolates showing maximum enzyme activities were maintained to utilize for further studies. The efficiency of enzyme production was calculated as the ratio of diameter of zone of clearance to colony diameter.

Characterization of bacterial isolates: The bacterial isolates with prominent zone of clearance and showing efficient enzyme production were processed for the determination of colony morphology, Gram staining, motility, biochemical tests and enzyme profiles then identified in accordance with the Bergey's Manual of Determinative Bacteriology. Further isolates were confirmed by VITEK 2 system version: 05.02.

Optimization of physicochemical parameters: Extracellular alkaline protease production was optimized for various physicochemical parameters[15]. The culture medium CYP was supplemented with different concentrations of nitrogen sources i.e. yeast extract and peptone; the varied incubation periods were 12, 24, 48, 72 and 96 hrs [16], temperatures 30, 35, 40, 45, 50,55^{0}C [17,18,19] and pH in the range of 7.0 to 11.0[4, 20] were considered. The fermentation experiment for the production of extracellular alkaline protease by isolates *B.pucilum* p1 and *S.auricularis* p18 were performed by inoculating 2% culture into 150ml culture medium in baffled Erlenmeyer flask at temperature 45^{0}C and agitation speed of 120 rpm for 96 hrs. Estimation of protease was done by standard protease assay[7,8].

Preparation of purified enzyme: The culture supernatant was obtained by centrifugation of culture broth at 10,000 rpm for 15 min at 4^{0}C. Ammonium sulphate was added into the culture supernatant and the precipitate obtained at 75% saturation was collected and dialysed.

Applications of alkaline proteases: Dehairing of hide: Goat skin was selected for a study which was washed with water to remove salt and other debris[1]. It was cut into the small pieces which were latter treated with enzyme. The skin pieces were treated with either crude enzyme preparation only (treatment 1); 7% sodium sulphide, lime and crude enzyme preparation (treatment 2); and 14% sodium sulphide and lime (Treatment 3) as positive control.

The skin pieces after above treatment were examined for depilation time, depilation extent, pelt colour and scud to evaluate the process of depilation with different depilating agents. The so produced crude material was further processed by conventional methods. The finally prepared pieces of leather were also accessed by examining the features such as colour, scud removal and general appearance of the body[5].

Bio-film degradation: Biofilm formation and quantification using glass test tubes were measured. Briefly, overnight cultures of *E.coli* and *P.fluroscence,* 0.2ml inoculated in biofilm growth medium and incubated overnight at 30^{0}C[10].

To screen for the efficiency of enzyme in removing the biofilm; the enzyme treatment was given in two ways such as in the medium during the incubation, and after the incubation at 45^{0}C.

After incubation the growth medium was gently removed by adding 2ml of cleaning solution (45^{0}C water was used as control) and incubated the tubes for 30 minutes. After which the solution was gently pipetted out. Than 2ml water and 2 µl of 1% crystal violet solution was added in each tube and incubated for 15 minutes. Later the tubes were rinsed three times with room temperature water. Crystal violet stained biofilms were solublized in 95% ethanol and thus formed crystal violet ethanol solutions were measure for absorbance at 595nm[21] .

Recovery of silver from waste X-ray photographic film: Used X-ray film was washed with distilled water and wiped with cotton impregnated with ethanol. The washed film was dried in an oven at 40˚C for 30 min. One g of X-ray film (2 x2 cm pieces) was then incubated in 10 ml of purified protease at 40˚C, pH-10 with continuous shaking. After complete gelatine hydrolysis reaction slurry contained gelatine and silver[9]. The obtainedslurry was dried and smelted in the presence of borax at 1100^{0}C in the furnace. The purity of the recovered silver was determined potentiometrically.

Results and discussion

Screening: The isolation and screening of alkaline protease producing bacterial isolates were done on the basis of mean value of zone of gelatin hydrolysis on gelatin agar medium (table 1).

Table-1
Screening of efficient alkaline protease producing isolate on the basis of zone of clearance

Isolates	Zone dia (mm)	Colony dia (mm)	Efficiency
P1	22	3	7.33
P4	26	5	5.2
P5	29	6	4.83
P12	27	10	2.7
P16	27	6	4.5
P18	36	17	2.11

Characterization and identification: The selected organisms were characterized on the basis of morphological, biochemical and enzymatic profile as per Bergey's manual of systematic bacteriology 4th edition. Further the species were identified on VITEK-2 system version:05.02 and confirmed as *Bacillus pumilus* p1 and *Staphylococcus auricularis* p18 (table 2).

Optimization of physicochemical parameters: The following parameters investigated for their effect on protease production are described below.

Incubation period: The maximum biosynthesis of proteases was observed at 72 hrs for both *B.pumilus* P1 and *S.auricularis* P18 (figure 1).

The production of protease by *B.pumilus* P1 was proportionally increased with the incubation up to 72 hrs but later it decreased dramatically.

Incubation Temperature and pH: The optimum temperature and pH for *B.pumilus* was observed to be 50^0C and 8.0, whereas for *S.auricularis* it was 45^0C and 9.0. Comparatively *S.auricularis* was found to be more stable at high alkaline pH values but the overall enzyme production by *B.pumilus* was high (figure 2 and figure 3).

Effect of nitrogen sources: The effects of different nitrogen sources viz. peptone and yeast extract in concentration of 0.1, 0.3, 0.5, 0.7 and 0.9% on protease production were investigated. The optimum values of N-sources for protease production are shown in figure 4. The influence of yeast extract was found to be more significant than peptone for both the organisms.

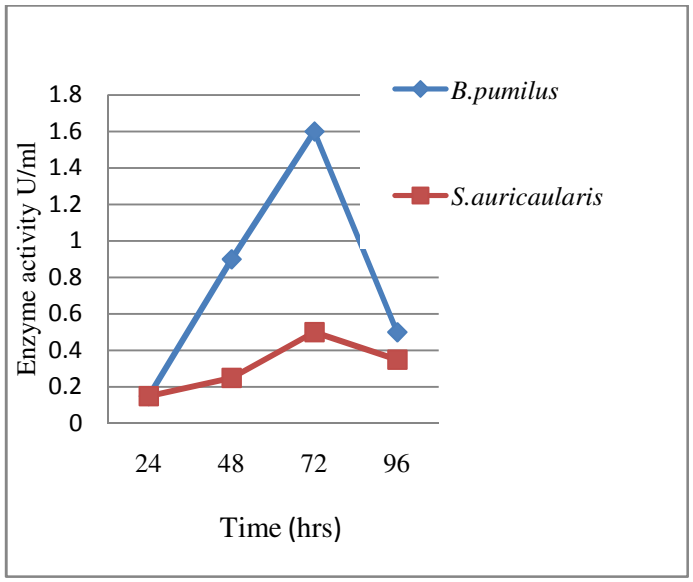

Figure-1
Effect of incubation period on enzyme production

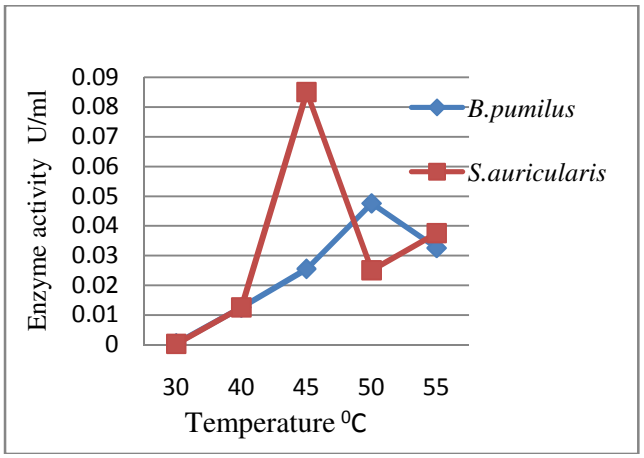

Figure-2
Effect of temperature on enzyme production

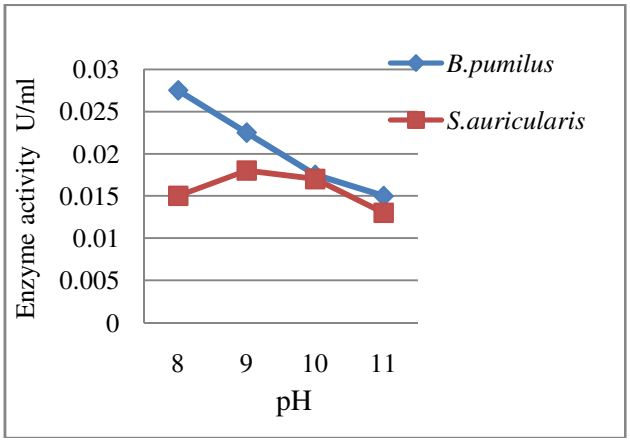

Figure-3
Effect of pH on enzyme activity on protease production

Table-2
Identification of *Bacillus pumilus* by VITEK-2 syatem version: 05.02

Bac-test laboratory			
bioMerieux Customer System #	Laboratory Report		Printed Apr. 2. 2012 07:25 IST Printed by bactest Patient ID:IS/1203/114 Bench: AIR SAMPLE
Bionumber: 1253105614566260 Selected organism: Bacillus pumilus			
Identification information	Card: BCL	Lot Number: 239213510	Expires: Oct 18, 2012 12.00
	Completed: Mar 24, 2012 03:01 IST	Status: Final	Analysis Time: 14.25 hours
Selected Organism	94 % Probability Bionumber: 1253105614566260	Bacillus pumilus	
			Confidence: very good identification

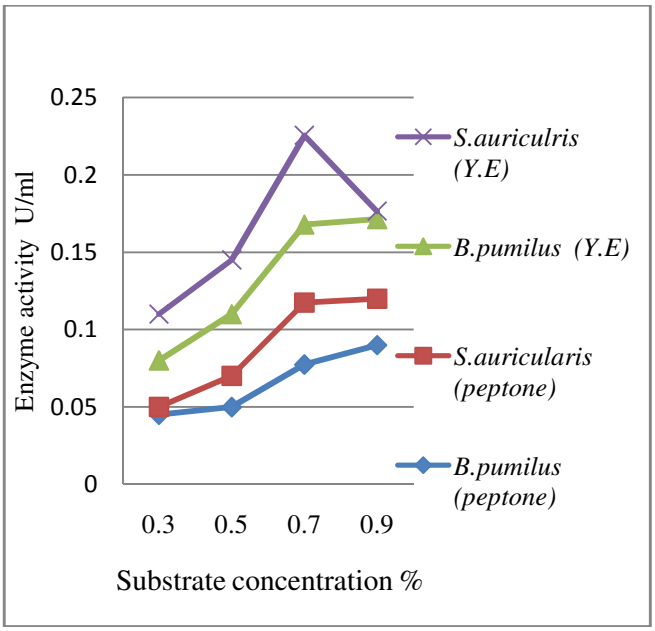

Figure-4
Effect of nitrogen sources on enzyme production

Applications: Leather processing: The physical and gross evaluation of the finally prepared leather revealed that it had grayish blue color, rough surface, fair stretch ability, too much scud and normal in appearance (table 3 and 4). Hence the purified enzymes were efficient in leather processing. The complete depilation was observed within 12 hrs.

Table-3
Evaluation of the pelt after treatment with alkaline proteases

Experiment	Time of depilation (hrs)	Scud	Pelt colour
Treatment1	12	+ +	White
Treatment2	07	+	White
Treatment3	05	+++	Blackish

Table-4
Evaluation of quality of the finally prepared leather treated with alkaline proteases

Experiment	Colour	Grain	Scud
Treatment 1	Grey	Smooth	Normal
Treatment 2	Grey	Smooth	Normal
Treatment 3	Grey-blue	Rough	Normal

Bio-film degradation: Purified enzymes of both the isolates were very effective in biofiln degradation. Enzyme of *B.pumilus p1* degraded 86% of the biofilm while *S.auriculais* was 50% efficient in degradation of biofilm (table 5 and 6).

Table-5
Efficiency of purified enzymes in removal of biofilm developed by *p. fluroscence*

Treatment by enzyme	*B.pumilus*	*S.auricularis*
Positive control	0.33	0.36
During incubation	0.27	0.29
After incubation	0.11	0.23

Table-6
Efficiency of purified enzymes in removal of biofilm developed by *E.coli*

Treatment by enzyme	*B.pumilus*	*S.auricularis*
Positive control	0.36	0.32
During incubation	0.33	0.25
After incubation	0.10	0.23

Silver recovery: Purified enzymes of both the isolates were very efficient in gelatine hydrolysis from waste x-ray photographic film. Under the obtained conditions (pH=8.0 and 40^0C), 0.4013 gm and 0.3823 gm silver was recovered by *B.pumilus* and *S.auricularis* respectively.

Conclusion

The extracellular alkaline protease producers were isolated, screened and identified successfully as *B.pumilus* and *S.auricularis.* The fermentation conditions were optimized in order to produce maximum alkaline proteases with high activity from both the strains.

The maximum enzyme activity at optimum temperature, pH and nutrient availability was 0.065U/ml for *B.pumilus* p1 and 0.038U/ml for *S.auricularis* p18. Enzymes isolated from *B.pumilus* p1 and *S.auricularis* p18 showed efficient activity in leather processing, biofilm degradation, hydrolysis of gelatine and recovery of silver from waste X-ray photographic film. These isolates and purified alkaline proteases enzymes may further be exploited for various industrial applications.

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