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Eco-Friendly Applications of Bacterial Extracellular Alkaline Protease

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Abstract: *Among the most important hydrolytic enzymes microbial alkaline protease have been extensively used since the advent of enzymology. It is produce by bacterium either extracellular or intracellular. Isolation and screening of sufficient extracellular alkaline protease producing bacterial strains are of great importance due to its wide spectrum applications in number of industries such as bio-remediation, food industries, leather processing, bio-film degradation, keratin degradation antifungal activity and number of other eco-friendly applications. The various samples, sea water, domestic water sludge, fish market soil and fermented fish were collected and used for enrichment, isolation and screening was done using screening medium and optimization for different physical and chemical parameters were carried out using CYP agar medium.*

Out of 18 isolates 16 were alkaline protease positive out of those; 4 isolates were able to produce sufficient amount of extracellular alkaline protease. The ratio of zone diameter to colony diameter from 4 isolates ranges from 3.11 to 8.41. Out of four efficient alkaline protease producers B.pumilus P1 showed significant enzyme activity. Optimizations of physical parameters were determined and found to be 72 hours of incubation period, 8.0 to 9.0 pH, 45^{0}C incubation period. Optimization of chemical parameters were determined for variety of carbon and nitrogen sources, were found to be 1.5% of glucose, lactose, 2% of sucrose,0.7% of ammonium sulphate, gelatin in case of refined sources. Wheat bran, sugarcane baggase as carbon sources and soya bean meal, cotton stalk gave maximum enzyme activity at 10% concentration. Enzyme obtained from refined sources and crude sources having maximum enzyme activity 16.5U/ml and 8.3U/ml respectively.

Crude as well as partially purified enzyme preparations were found to be excellent in dehairing, depilation, of raw leather, degradation of feather, bio-film degradation and antifungal activity.

Keywords: *Alkaline protease, Leather processing, Antifungal, Insecticidal, Bio-film, B.pumilus*

I. Introduction

Proteases are the enzymes occur anywhere in nature. They are found to be inside or on the surface of the organism[1]. Proteases break down protein by hydrolysis of peptide bond that exists between two amino acids of polypeptide chain. Today proteases available in the market are derived from microbial source[2].

Proteases are classified according to their active pH range in to Neutral, Acidic and alkaline. Proteases from marine microbes are currently receiving increasing attention due to their inherent stability at different values of pH, temperature and salinity[3, 4, 5].

Proteases are having number of environmentally friendly applications. It can substitute number of chemical fungicides[6], insecticides in agriculture[6], dehairing agents in leather processing[8,9], it removes bio-film very efficiently[4], control water and soil pollution.

Proteolytic enzymes are more efficient in enzymatic dehairing of raw leather than amylolytic enzymes. They are also useful in detergent formulations[9].

II. Materials And Methods

Sample: Soil samples from vicinity of mutton market, fish market, similarly waste water sludge, fermented fish and sea water were used to prepare initial inoculums.[2,10]

Isolation: The sample from different locations inoculated in 100ml nutrient broth; pH value ranging from 8-10 and enrichment is carried out for 48 hrs at room temperature under shaking condition.[2,10]

Enriched broth was diluted and spread plated on skimmed milk agar medium consisting of each (%W/V) skimmed milk powder (5), peptone (0.2) NaCl (0.5) agar (2) and pH 8-10. Plates were incubated at room temperature and examine for halo around the colonies as the sign of protease producing ability of an organism[11]. Based on zone of casein hydrolysis different isolates were selected and were maintained on agar plats[11].

Screening: A suitably diluted culture 0.2ml was spread on casein yeast extract peptone agar medium or on nutrient agar medium with 4% gelatin was inoculated at 37^{0}C for 24 hrs; plates were flooded with 1% tannic acid solution colonies showing clear zone of hydrolysis were picked and purified[12].

Enzyme activity was calculated as the ratio of diameter of zone of clearance to colony diameter[12].

Characterization of bacterial isolates: Bacterial isolates with prominent zone of clearance and showing efficient enzyme production were processed for determination of colony morphology. Gram staining, motility, biochemical test and enzyme profile then identified in accordance with Bergey's manual of determinative bacteriology, further isolates were confirmed by VITEK-2 system version 05.02.

Optimization of physic-chemical parameters: Extracellular alkaline protease production was optimized for various physico-chemical parameters. The production medium CYP was supplemented with different concentration of refined nitrogen sources, carbon sources[8] and crude nitrogen, carbon sources[2,17]. The various incubation periods were 12, 24,48,72 and 96 hours, Temperature 30,35 40,45,50 and 55^{o}C[8,9], pH values ranged from 7.5 to 10.5[13,14] and different sodium alginate concentration in case of immobilized cell.

Fermentation experiment was carried out for production of extracellular alkaline protease by isolate *Bacillus pumilus* p1. The enzyme was produced by inoculating 2% culture in to 200ml production medium in bottled Erlenmeyer flask at its optimized conditions[2]. Enzyme production was carry out using submerged and solid state fermentation using refined and crude sources respectively[1,4,20].

Estimation of protease was done by standard protease assay.

Preparation of purified enzyme: The culture supernatant obtained by centrifugation of cultured broth at 10,000rpm for 15 min's at 4^{0}C. Ammonium sulphate was added in to culture supernatant and the precipitate is obtained at 60-70% saturation was collected and dialyzed[3,13].

Applications of alkaline protease: Dehairing of hide: Goat skin was selected for a study which was washed with water to remove salt and other debris. It was cut in to small pieces which were later 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[8,9].

The skin pieces after above treatment were examined for depilation time, depilation extent; pelt color and scud evaluate the process of depilation with different depilating agents. So, the 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.

Keratin degradation: Keratin degradation was observed using enzymatic digestion of feather and biological degradation of chicken feathers. In enzymatic digestion feathers were treated with purified enzyme preparation. In biological degradation feathers were directly incubated with isolated microbial cells with negative and positive controls. The use of enzymes in feather meal is an attractive and alternative approach over conventional methods which results in loss of essential amino acids.

The ability of bacillus strain to produce appreciable level of keratinase and protease using feather as substrate could open new opportunities for the achievement of efficient biodegradation. Keratin degradation using microbes helps to reduce environmental impact of chemicals.

Bio-film degradation: Bio-film formation and quantification using glass taste tubes were measured. Overnight culture of *E. coli* and *pseudomonas fluorescence*, 0.2 ml inoculated in biofilm growth medium and incubated overnight at 37^{0}C[4].

To screen for the efficiency of enzyme in removing 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 is used as control) and incubated the tubes for 30 minutes. After which the solution was gently pipetted out. Then 2ml water and 2µl of 1% crystal violet solution was added in each tube and incubated for 15 minutes. Later tubes were rinsed with normal water. Crystal violet stained bio-film was solubilized in 95% ethanol and thus formed crystal violet ethanol solution were measured for absorbance at 595 nm[4].

Antifungal activity

Test organism: Two fungal plant pathogens were isolated from local soil sample, were grown on potato dextrose agar plates and incubated at 30^{0}C for 5 days. The isolated fungal plant pathogen cultured on slants of PDA and preserved for further study[19].

Pure cultures of fugal plant pathogens were identified as *Fusarium solani* and *Rizoctonia solani* according to some cultural properties and morphological characterstic's.

Assay of antifungal activity: Antifungal activity of partially purified protease enzyme against *F.solani* and *R.solani* was carried out using agar well diffusion technique using 100µl partially purified enzyme. 1ml of test culture was used for surface inoculation on PDA and antifungal activities were determined after 4 days of incubation at 30^{o}C by measuring diameter of developed inhibition zone[6].

III. Results And Discussions

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 isolates on the basis of zone of clearance.

Isolates	Zone dia(mm)	Colony dia (mm)	Efficiency
p1	24±0.8	2±0.6	12.0
p2	28±1.2	4±0.5	7.0
p3	29±0.5	7±0.3	4.1
p4	38±0.3	15±0.2	2.5

Characterization and identification: The selected isolate was characterized on the basis of morphological, biochemical and enzymatic profile as per Bergey's manual of systematic bacteriology 4th edition. Further the species was identified on VITEK-2 system version 05:02 and confirmed as *Bacillus pumilus* p1.

Optimization of physicochemical parameters: The following factors investigated for their effect on protease activity are incubation period, incubation temperature and various pH values. As well as different refined and crude carbon, nitrogen sources were optimized for enzyme activity. Effect of immobilized cells on enzyme activity was found out using different concentrations of sodium alginate.

Table 2. Identification of alkaline protease producing organism using VITEK 2 system version: 05.02

Bac-test laboratory — Printed Dec. 2. 2016 07:25 IST Bio Merieux Customer — Laboratory Report
Printed by bactest System # — Patient ID:IS/1203/114 — Bench: AIR SAMPLE

Bionumber: 1253105614566260
Selected organism: Bacillus pumilus

Identification information — Card: BCL — Lot Number: 239213510 — Expires: Oct 18, 2017 12.00
Completed: Dec 3, 2016 03:01 IST — Status: Final — Analysis Time: 14.25 hours

Selected Organism Bionumber: 1253105614566260 — 94 % Probability — Bacillus pumilus — Confidence: very good identification

Contraindicating Typical Biopattern(s)
Bacillus pumilus BNAG (92), LeuA (79), APPA(17), BMAN(22).

Biochemicaldetails:

1	BXYL	+	3	LysA	-	4	AspA	(-)	5	LeuA	-	7	PheA	+	8	ProA	-
9	BGAL	+	10	PyrA	-	11	AGAL	+	12	AlaA	+	13	TyrA	+	14	BNAG	-
15	APPA	+	18	CDEX	-	19	dGAL	-	21	GLYG	-	22	INO	-	24	MdG	(-)
25	ELLM	+	26	MdX	-	27	AMAN	+	29	MTE	-	30	GlyA	(+)	31	dMAN	+
32	dMNE	+	34	dMLZ	-	36	NAG	-	37	PLE	-	39	IRHA	-	41	BGLU	+
43	BMAN	+	44	PHC	-	45	PVATE	+	46	AGLU	-	47	dTAG	+	48	dTRE	+
50	INU	-	53	dGLU	+	54	dRIB	+	55	PSCNa	-	58	NaCl 6.5%	+	59	KAN	-
60	OLD	-	61	ESC	+	62	TTZ	+	63	POLYB_R	-						

Installed VITEK 2 System Version: 05:02
MIC Interpretation guideline: — Therapeutic Interpretation guideline:
AES Parameter Set Name: — AES Parameter Last Modified:

Effect of Incubation period on enzyme production: The maximum biosynthesis of proteases was observed within 72 hours using *B.pumilus* p1. The production of proteases was proportionally increased with the incubation time within the time range of 12 to 96 hours of incubation, whereas after 72 hours of incubation the protease activity decreased considerably (Figure 1).

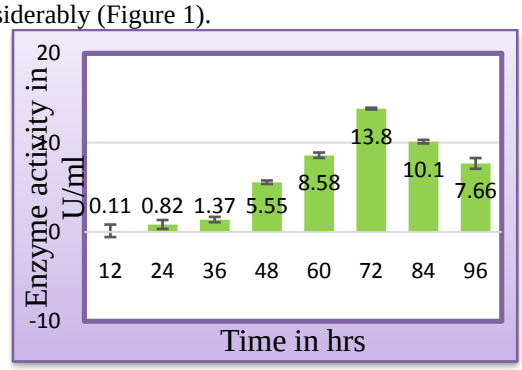

Figure 1.Effect of incubation period on enzyme activity.

Effect of temperature on enzyme production: The result of effect of different incubation temperatures on production of protease by bacterial isolate was shown in Figure 2. The maximum temperature for *B.pumilus* P1 for biosynthesis of alkaline protease was 45^0C.

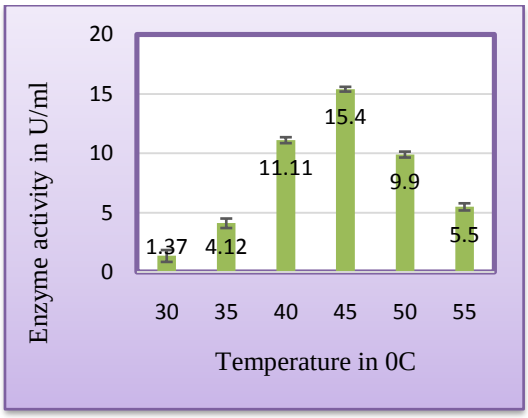

Figure 2.Effect of incubation temperature on enzyme production.

Effect of pH on enzyme production: The optimum pH at which *B.pumillus* p1 showed maximum production is shown in Fig 3. The optimum pH value for alkaline protease production by *B.pumilus* P1 was 8.5.

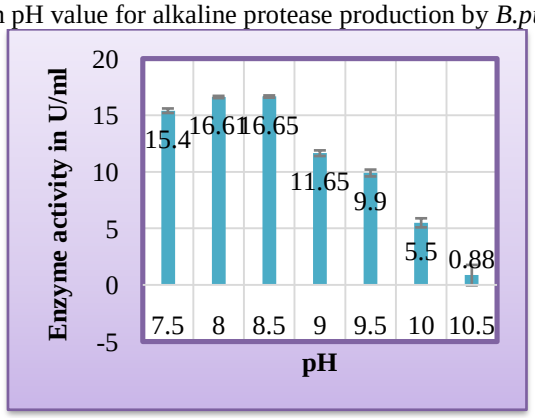

Figure 3. Effect of pH on enzyme activity on protease production

Optimization using immobilized cells using sodium alginate: The optimum sodium alginate concentration at which *B.pumillus* p1 showed maximum activity is shown in Fig 4. The optimum sodium alginate concentration value for *B.pumilus* P1 was 2.5%.

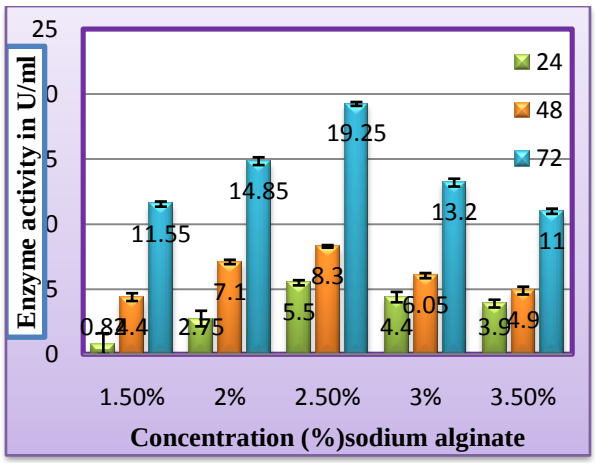

Figure 4.Effect of sodium alginate concentration (%) on enzyme activity (U/ml) at different incubation period.

Effect of nitrogen sources on enzyme production: The effect of different refined and crude nitrogen sources shown in figure 5. Among different crude nitrogen sources soyabean meal was excellent in alkaline protease production using *B. pumilus* p1 respectively.

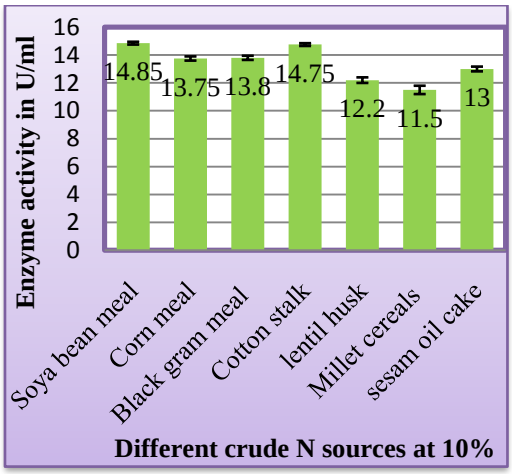

Figure 5. Effect of crude nitrogen sources on alkaline protease production.

Alkaline protease enzyme produced from refined and crude substrates gave 16.5U/ml and 8.3U/ml enzyme activity respectively as shown in figure 6.

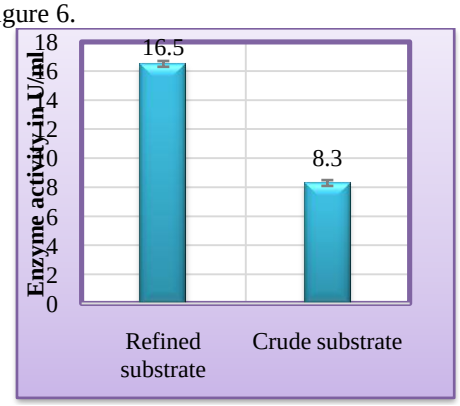

Figure 6. Enzyme activity of crude enzyme extract obtained from refined substrate and crude substrate

Applications: Leather processing: The results of the physical tests showed that the gross evaluation of the finally prepared leather. It had grayish blue color, rough surface, fair stretch ability, too much scud and a normal appearance. (Table 3 and 4) The crude enzymes are efficient in leather processing. The complete depilation was observed in 12 hrs.

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

Experiment	Time of depilation (hrs)	Scud	Pelt colour
Treatment 1	10	++	White
Treatment 2	7	+	White
Treatment 3	5	+++	Blakish

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

Experiment	**Colour**	**Grain**	**Scud**
Treatment 1	Grey	Smooth	Normal
Treatment 2	Grey	Smooth	Normal
Treatment3	Grey -blue	Rough	Normal

Keratin degradation: The *B.pumilus* p1 strain was able to grow after 2 days culture in mineral medium containing 30 grams chicken feather as sole source of carbon, nitrogen and sulphur.

Intense feather degrading activity was achieved at 45^0Cand at pH 8. Nearly complete feather degradation observed at the end of 72 hrs incubation. In contrast no degradation was noted with control.

Furthermore, when chicken feathers were incubated only with purified enzyme total degradation was observed within 24 hrs. Therefore, the use of enzymatic and microbiological method for hydrolysis of feather is an attractive alternative to currently used method of feather meal preparation.

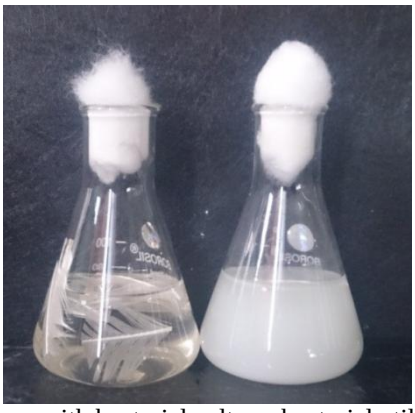

Figure 7. After incubation of feathers with bacterial culture, bacterial utilize feather as carbon and nitrogen sources and show degradation in 24 hrs. Control (left flask), Test (Right flask).

Bio-film degradation: purified enzyme of the isolate *B.pumilus* p1 was very efficient in removal of bio-film (table 5).

Table -5. Efficiency of purified enzyme in removal of bio-film developed by *P.fluroscence* and *E.coli*.

Treatment by enzyme	*P. fluroscence*	*E.coli*
Positive control	0.35	0.37
During incubation	0.30	0.29
After incubation	0.10	0.10

Antifungal activity: The result from the well diffusion technique showed clear inhibition zone of 3.5mm diameter, confirmed the antifungal activity of purified protease. The potential of the purified enzyme to inhibit *F.solani* and *R.solani* growth in vitro condition indicates that this enzyme may play important role in controlling fungal plant pathogens.

Conclusions: The extracellular alkaline protease producer was isolated screened and identified successfully as *B.pumilus*. The fermentation conditions were optimized in order to produce maximum alkaline protease with high activity.

The maximum enzyme activity at optimum incubation period, temperature, pH, nutrient availability of refined sources and crude sources was 16.5U/ml and 8.3U/ml respectively. Enzyme isolated from *B.pumilus* showed efficient activity in leather processing, keratin degradation, Bio-film removal, and Antifungal agent of fungal plant pathogen. This isolate and purified alkaline protease enzyme may further be exploited for various industrial applications.

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