Isolation of Thermos table Extracellular Alkaline Protease and Lipase Producing Bacteria from Tannery Effluents

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Abstract --- The aim of the present study was to isolate the extracellular protease and lipase producing bacteria from tannery effluents. The bacterial isolation was performed by serial dilution and plating method. Total of eight protease producing isolates were screened on the basis of their clear zone formation on skim milk agar. Further screening for lipase producing stains on tributyrin agar confirmed that only four were able to produce an extracellular lipase enzyme. Isolated bacteria were identified on the basis of morphological, cultural, and biochemical characterization. Among four promising isolates three were belong to the genus *Bacillus spp* while one was identified as *Actinobacillus* spp.

Keywords--- Tannery Effluent; *Bacillus; Actinobacillus*; Extracellular Protease, Lipase

I.INTRODUCTION

Industrial pollution is one of the biggest contributors to pollute our environment including land we live on, the water we use and the air we breathe. It is one of the major factor causing the degradation of the environment around us and ultimately affects the ultimately affects the livelihood in the affected areas [1].

The environment is under increasing pressure from solid and liquid wastes emanating from the leather industry. Effluent from leather Industry is considered as a major contributor in the industrial pollution and the pollutants causing environmental hazards includes viz. lime, sodium sulphide, salt, solvents, etc. which aroused mainly from the pre-tanning processes of leather processing [2]. Tannery effluents are documented to be responsible for the highest pollutants among all industrial wastes. The major contribution of chromium pollution is a resulted due to the discharge of the untreated tannery effluents.

In India alone about 2000–3000 tone of chromium escapes into the environment annually from tannery industries, with chromium concentrations ranging between 2000 and 5000mg/L in the aqueous effluent as compared to the recommended permissible discharge limits of 2 mg/L [3].

Proteases are complex group of enzymes collectively known as peptidyl-peptide hydrolases and are responsible for hydrolysis of peptide bonds in a protein molecule. Application of proteases is predominant across several industrial segments including leather, detergent, food, meat tenderization, textiles and pharmaceuticals [4].

Lipase enzymes are involved in the breakdown and mobilization of lipid and are being widely used in detergents, degradation of leather and dairy effluent, baking and pharmaceutical industries [5]. In leather industry, in view of eco-friendly industrial processes, use of proteases is increasing in pre-tanning processes of leather. This eco-friendly process also avail several advantages, as easy process control, speed and waste reduction along with economical gain [6].

Microbial diversity is a major resource for biotechnological products and processes development. The multitude of physicochemically diverse habitats has challenged nature to develop equally numerous molecular adaptations in the microbial world. The microorganisms from diverse environments called as extremophiles, are an important source of producing novel enzymes with better properties and suitable for commercial exploitation, whose specific properties are likely to result in novel applications [7].

The use of microorganisms, microbial process, or microbial products to detoxify and degrade the pollutants in the effluents is one the emerging technologies known as Biodegradation. Different microbes producing enzymes are used for the effluent remediation process [8]. The aim of the present study was to search a thermo stable, extracellular protease and lipase producing bacteria from tannery effluents.

II. MATERIALS AND METHODS

A. Sampling area and sample collection

Samples of tannery effluent were collected from the two different sites of G & G Organics Application Lab, India. Duplicate sample of the liquid effluent was collected in sterile bottle and transported to the

laboratory maintaining temperature around 4˚C as early as possible for microbiological analysis.

B. Isolation of extracellular protease producing bacteria

10 mL of effluent samples were added to 90 mL of sterile water in an Erlenmeyer flask. Subsequently, 1 mL of the suspension was added to 9 mL of sterile saline water and a serial dilution (10^{-1} to 10^{-6}) was prepared. 1 mL of each dilution was inoculated onto skim milk agar plates. Plates were incubated at 50°C for 24-72 h. Formation of clear zone around the colonies, resulting from casein hydrolysis, was taken as evidence of protease enzyme producers as a result of proteolytic action. Isolation methodology was based on the earlier studies [9] being applied for the isolation of thermo stable protease producing bacteria from tannery effluent. Among the clear zone forming colonies, only larger zone forming colonies were selected for purification and further study.

C. Purification of the isolates and maintenance

The isolated organisms were purified through repeated subculture method. Streak plate method was used with nutrient agar as growth media. When a plate yielded only one type of colony, the organisms were considered to be pure. The purification of the isolates was also confirmed by microscopic observation. The pure cultures so obtained were maintained on nutrient agar slants.

D. Identification of extracellular lipase producers

The isolated protease producing cultures obtained in the pure cultures were inoculated in nutrient broth and further (1mL) on medium containing (g/L): beef extract 3.0, peptone 5.0, sodium chloride 5.0, agar 15.0, calcium chloride 0.05 and glycerol tributyrate 0.2 mL. Plates were incubated at 50°C for 24-72 h. Formation of clear zone around the colonies, resulting from lipid hydrolysis, was taken as evidence of lipase enzyme producers as a result of lipolytic action [10].

E. Cultural condition for the production of alkaline protease

A loopful of the selected isolate from the pure culture was inoculated into 100 mL-glass tube containing 10 mL of alkaline protease producing medium and incubated overnight at 50°C and 180 rpm in a rotary shaker incubator. 5 mL of this culture was then inoculated into 500 mL capacity Erlenmeyer flask containing 95 mL of the same medium and incubated at 50°C in an orbital shaker for 72 h. Cells and insoluble content of the medium were removed by centrifugation at 10000 g for 10 min at 4°C and the cell free supernatant was membrane filtered (0.2-µm pore-size) which was used as the source of crude protease enzyme.

F. Alkaline protease assay

Protease activity was determined as described earlier [11]. The reaction mixture containing 2 ml of 1% casein solution in 50 mM glycine- NaOH buffer (pH 11) and 1 mL of crude enzyme solution were incubated at 60 °C for 15 min and the reaction was then stopped or terminated with the addition of 3 ml of 10 % trichloroacetic acid (TCA). After 10 min the entire mixture was centrifuged at 10000 g for 10 min at 4 °C and the absorbance of the liberated tyrosine was measured with respect to the blank (non-incubated sample) at 280 nm. One protease unit was defined as the amount of enzyme that releases 1 µg of tyrosine per mL per minute under the defined assay conditions. Standard curve of tyrosine was done using 1, 2, 4, 8, and 16 µg/mL tyrosine in 50 mM glycine-NaOH buffer, pH 11.

G. Cultural condition for the production of Lipase

A loopful of culture producing lipase from agar plate was inoculated into 100 ml-glass tube containing 10 mL of basal medium with 2% olive oil for production of lipase and incubated overnight at 50 °C and 180 rpm in a rotary shaker incubator. 5 mL of this culture was then inoculated into 500 mL capacity Erlenmeyer flask containing 95 ml of the same medium and incubated at 50 °C in an orbital shaker for 72 h. Cells and insoluble content of the medium were removed by centrifugation at 10000 g for 10 min at 4 °C and the cell free supernatant was membrane filtered (0.2-µm pore-size) which was used as the source of crude lipase enzyme [8].

H. Lipase assay

Lipase assay was carried out based on the earlier studies [12]. Reaction mixture with 2% of olive oil substrate solution (27 mL) and 20 mL of the crude enzyme solution was continuously stirred on magnetic stirrer for 30 min. After stirring the mixture, 2-3 drops of phenolphthalein indicator was added and it was titrated against 1 N NaOH solution. The end point was colorless to pink. Units of lipases were calculated in terms of fatty acids produced per mL which were calculated in terms of acetic acid released in the respective reaction mixture under defined set of assay condition.

I. Identification of the bacterial isolates

Different morphological and biochemical analysis was performed and each isolate was characterized and identified based on Bergey's manual of determinative bacteriology [13].

III. RESULTS AND DISCUSSION

A. Isolation and screening of thermo-stable extracellular alkaline protease producing bacteria

For the isolation of thermo-stable extracellular alkaline protease producing bacteria from the tannery effluent, the collected effluent sample was serially diluted and preliminary inoculated on skim milk agar media. Bacterial colonies with larger clear zones (Fig.1) were confirmed as protease producers. Clear zones were formed as a result of the hydrolysis of casein by protease produced from the isolates. Since the fat content of the whole milk inhibits the growth of bacteria, skim milk was used during the present study. Total of eight (I-1 to I-8) bacterial isolates were selected as a promising protease producers.

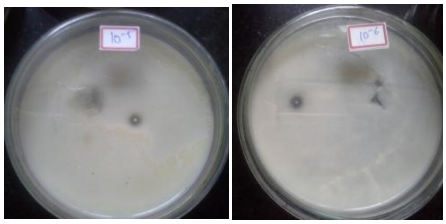
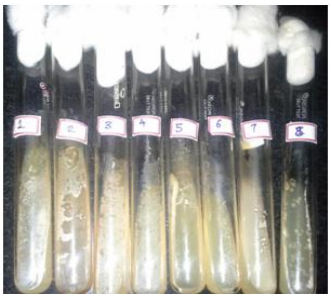


Fig 1 Clear zone of *Protease producing bacterial isolates on Skim milk Agar*

B. Pure culture of the Bacterial Isolates and Maintenance

Subcultures were performed on nutrient agar slant for each of the isolate obtained from uniform colonies and total of eight pure cultures were stored at 4 ˚C for further work.



. Fig.2 Pure cultures of the Protease producing Bacterial Isolates

C. Isolation and screening of thermo-stable extracellular Lipase producing bacteria

Total of eight bacterial isolates producing extracellular protease were subjected for their ability to produce extracellular lipase. Out of eight isolates only four bacterial isolates (I-1, 1-4,I-7 and I-8) were observed to produce the lipase enzyme (Table 2) with clear zone as a result of their lipolytic activity.

TABLE 2
ISOLATED BACTERIAL STRAINS AND ENZYME PRODUCTION CAPABILITY

Bacterial Isolates	I-1	I-2	I-3	I-4	I-5	I-6	I-7	I-8
Protease Enzyme	+	-	+	+	-	+	+	+
Lipase Enzyme	+	+	-	+	-	-	+	+

(+) Present; (-) Absent

D. Confirmation of promising thermo-stable extracellular Lipase and alkaline protease producing isolate

Four isolates (I-1, 1-4, I-7 and I-8) which were confirmed for their extracellular protease and lipase enzyme producing capability were further subjected for the production of enzymes followed by the respective enzyme assays. Among all the selected bacterial isolates, the isolate I-4 was observed to be the most promising protease (9 U/mL) and lipase (8 U/mL) producing bacterial strain. The isolate I-8 observed to produce maximum units of protease enzyme (10 U/mL), however the lipase produced was observed to be (4 U/mL) respectively. The isolate I-1and I-7 were showed comparatively the minimum enzyme producing capability.

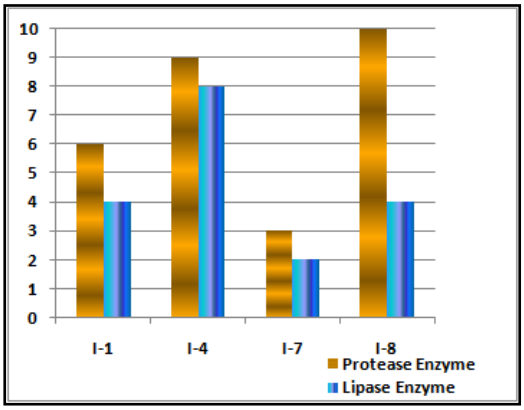


Fig.3 Comparative Enzyme Production among the selected Bacterial Isolates

E. Identification of the promising bacterial isolate

In the present study, identification of the most promising thermo-stable extracellular lipase and alkaline protease producing isolates was performed traditionally by isolating the organism and studying it cultural characteristics, morphological characteristics and biochemical characteristics. The results of these tests were correlated with the characteristics of bacteria described in Bergey's manual of determinative bacteriology [13]. Based on gram staining the bacterial isolates I-1, 1-4 and, I-7 were observed to be Gram positive rod, whereas the isolate I-8 was observed as a

Gram negative rod. The results obtained from the biochemical characterization test were summarized in the Table 3.

TABLE 3

CULTURAL AND BIOCHEMICAL CHARACTERS OF THE ISOLATES

Characteristics	I-1	I-4	I-7	I-8
Colony On Nutrient Agar	Creamy white, big, flat irregular	Creamy, slimy	Creamy, Irregular	Creamy white,Rhizoid, Irregular,Flat, Mucoid
Gram's Nature	Gram's Positive rod	Gram's positive rod	Gram's Positive rod	Gram's negative rod
Catalase Reaction	+	+	+	+
Aerobic Growth	+	+	+	+
Anaerobic Growth	-	-	-	+
Citrate Utilization	+	+	+	-
Lactose utilization	+	+	+	-
Dextrose	+	+	+	-
Mannitol	-	-	+	+
Spore Formation	+	+	+	-
Oxidase test	-	-	-	+
Triple sugar test	+	+	+	-
Sulphate Test	-	-	-	-

(+)Test is Positive ; (-) Test is Negative

The results divulge the identification of four isolates as I-1, 1-4, I-7 from the genus *Bacillus*, whereas the isolate I-8 was identified as the *Actinobacillus* spp. Among the microbial sources, bacteria especially *Bacillus* spp. have been exploited for the production of extracellular proteases and lipases [14]. Some earlier studies documented also confirmed the production of alkaline protease by *Actinobacillus* spp which secreted the metalloproteases into its medium [15].

IV. CONCLUSION

Today microbes are considered as one of the principal sources of industrial enzymes due to their simple mass culture techniques with extensive biochemically diversified media and technological developments in its genetic modifications.

The industrial effluents are enriched media to grow and spread microbial population. From the tannery effluent eight thermo-stable extracellular alkaline protease producing bacteria were isolated on the basis of formation of clear zone in skim milk agar. These isolates were further screened for their extracellular lipase producing capability on tributyrin agar. Out of total four lipase and protease producers, three bacterial isolates were identified as *B*acillus spp. whereas, one of the isolate was belong to the genus *Actinobacillus.* The most promising bacterial isolate i.e. I-4 from *B*acillus spp. showed the maximum lypolytic (8 U/mL) as well as high proteolytic activity (9 U/mL), compare to that of other isolates. Based on this analysis **the** isolate I-4 maximum enzyme activity will be significant for the future studies**.**

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