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ISOLATION, OPTIMIZATION AND CHARACTERIZATION OF ALKALINE PROTEASE PRODUCING BACTERIA FROM COLLAGEN LAYER OF DECAYING SKIN (SHEEP UPPER) LEATHER INDUSTRY

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ABSTRACT

Enzymes are organic catalysts that play a vital role in many aspects of life. Enzymes play a huge role in day today life from simple fermentation to complicated gene expressions. Protease is enzyme that has the ability to degrade protein by the breaking of the hydrogen bond that bunds protein into peptides and proteins. Microbial proteases belong to acid, neutral or alkaline based on their pH optimum for activity and the active site viz., Metallo (EC 3.4.24), Aspartic (EC 3.4.23) Cysteine or Sulphydryl (EC.3.4.22) or Serine type (EC 3.4.21). Alkaline protease is stable in alkaline pH and possesses a serine residue at the active site. Proteases constitute a class of industrial enzymes which alone form approximately 60% of the total world-wide enzyme production . Among the various proteases microbial proteases play an important role in biotechnological process. Alkaline proteases produced are of special interest as they could be used in the manufacture of detergents, food, pharmaceuticals and leather. Alkaline protease is produced by a wide variety of microbial species like *Bacillus subtilis*, *Aspergillus oryzae*, *Streptomyces cellulasae* and *Aeromonas hydrophila* species. Most commercial proteases, mainly neutral and alkaline are produced by organisms belonging to the *Bacillus* sp. *Bacillus* sp are attractive industrial tools for a variety of reasons, including their high growth rates leading to short fermentation cycle times, their capacity to secrete proteins into the extracellular media and the GRAS (Generally Regarded as Safe) status with food and drug administration for species such as *Bacillus subtilis* and *Bacillus licheniformis* . *Bacillus* sp is used extensively for the production of industrial enzymes such as amylase, lipase and alkaline protease. It is a popular host for industrial preparation of gene products. In our study

twenty one (21 No's) bacterial strains were isolated from the sheep upper collagen layer of decaying skin sample. The fifteen (15 No's) isolates exhibited proteolytic activity based on clear zone formation on skim milk agar medium at pH 9.0. These isolates were then characterized and identified. Biochemical test revealed the organisms to be *Bacillus* sp and *Pseudomonas* sp. Maximum yield of enzyme (22 mg/ml) was obtained in Naser Tanning company Chennai.

KEY WORDS: Alkaline Protease, Sheep Collage Layer, *Bacillus* sp.

INTRODUCTION

Enzymes are well known biocatalysts that perform a multitude of chemical reactions and are commercially exploited in the detergent, food, pharmaceutical, diagnostics, and fine chemical industries. Of the 3000 different enzymes described to date the majority have been isolated from mesophilic organisms. These enzymes mainly function in a narrow range of pH, temperature, and ionic strength. Moreover, the technological application of enzymes under demanding industrial conditions makes the currently known arsenal of enzyme not recommendable. Thus, the search for new microbial sources is a continual exercise, but one must respect biodiversity. The microorganisms from diverse and exotic environments, (Extremophiles) are considered as important source of enzymes, and their specific properties are expected to result in novel process applications (Govardhan and Margolin 1995)

Alkaline proteases (or Subtilisins, E.C. 3.4.21.14) are a physiologically and commercially important group of enzymes used primarily as detergent additives. They play a specific catalytic role in the hydrolysis of proteins. In 1994, the total market for industrial enzymes accounted for approximately $400 million, of which enzymes worth $112 million were used for detergent purposes. In Japan, 1994 alkaline protease sales were estimated at 15000 million yen (equivalent to $116 million) (Horikoshi 1996) . The use of alkaline proteases so that by the turn of the decade the total value for industrial enzymes is likely to reach $700 million or more (Hodgson 1994).

Alkalophilic Microorganisms

All microorganisms follow a normal distribution pattern based on the pH dependence for their optimal growth, and the majority of these microorganisms are known to proliferate well at near-neutral pH values. As the pH moves away from this neutral range, the number of microorganisms decreases. The number of alkalophilic bacteria found in the soil is about 1/10

to 1/100 of that of neutrophilic bacteria. However, some neutrophilic organisms are capable of growing even at extreme pH conditions. This is primarily due to the special physiological and metabolic systems, which they have adopted by altering the bioenergetics membrane properties and transport mechanisms, enabling their survival and multiplication under such adverse conditions (Krulwich 1986). Such microorganisms may also be referred to as pH-dependent extremophiles. Alkalophilic microorganisms constitute a diverse group that thrives in highly alkaline environments.

They have been further categorized into two broad groups, namely, alkalophiles and alkalotolerants. The thermo-alkalophiles is used for those organisms that were capable of growing above pH 10, with an optimal growth around pH 9, and are unable to grow at pH 7 or less. On the other hand, alkalotolerant organisms are capable of growing at pH values in excess of 10, but have an optimal growth rate nearer to neutrality (Krulwich 1990). The extreme alkalophiles have been further subdivided into two groups, namely, facultative and obligate alkalophiles. Facultative alkalophiles have optimal growth at pH 10 or above but can grow well at neutrality, while obligate alkalophiles fail to grow at neutrality (Krulwich and Guffanti 1989).

Habitat of Alkalophilic Microorganisms

Alkalophilic microorganisms are widely distributed in nature and can be found in almost all environments without the restriction of alkalinity. However, a few of the naturally-occurring alkaline environments, namely soda soils, lakes, and deserts, harbor a wide range of these types (Kumar *et al.,* 1997). Their ecological and chemical aspects have been studied in detail (Grant and Tindall 1986). Others include the dilute alkaline springs, desert soils and soils containing decaying proteins or forest soil (Durham1987). The pH values of these environments are commonly around 10 and above. The man-made alkaline environments were found to be the effluents from food, textiles, tanneries, potato processing units, paper manufacture units, calcium carbonate kilns, detergents and other industrial processes (Joshi and Bakk 1993). Highly saline, alkaline environments are relatively rare in the world compared with high saline, neutral environments. However, there is a possibility that such environments harbor a unique microbial population (Kostrikina *et al.,* 1991). The best sources for halophilic alkalophiles have been the extreme soda lakes of the Wadi Natrum in Egypt and Lake Magadi in Kenya (Grant and Tindall 1980). The study of this unique group of microorganisms has aroused interest because of the extreme tolerance of haloalkalophiles

to the two environmental extremes, salinity and high pH . Moreover, in this category there are also moderate thermopiles, with growth temperatures of approximately 40°C.

Isolation and Screening

The isolation of obligate alkalophilic organisms from human and animal feces was first reported by Vedder in 1934. He briefly described these organisms and proposed the name *Bacillus alcalophilus* for his strains and also stated that he had been able to prove that life exists that not only tolerates, but depends on, a highly alkaline pH (Vedder 1934) . Today, many of these alkalophilic *Bacillus* strains are of considerable industrial importance, particularly for use of proteases in laundry detergents (Aunstrup *et al.*,1972) , Xylanases for use in paper pulp industry (Nakamura *et al.*,1993), and cyclodextrin glucanotransferase for cyclodextrin manufacture from starch (Kitamoto 1992) . These industrial applications have prompted the isolation of alkalophilic microorganisms from a variety of natural and man-made alkaline environments (Jone *et al.,* 1994). Normal garden soil was reported to be a preferred source for isolation, presumably because of the various biological activities that generate transient alkaline conditions in such environments (Grant *et al.*,1990). These organisms were also isolated from non alkaline habitats such as neutral and acidic soils, and thus appear to be fairly widespread.

One of the most important and noteworthy features of many alkalophiles is their ability to modulate their environment. They can alkalinize neutral medium or acidify high alkaline medium to optimize external pH for growth. However, their internal pH is between pH 7 and 9, always lower than the external medium. Thus, alkalophilicity is maintained by these organisms through bioenergetic membrane properties and transport mechanisms, and does not necessarily rely on alkali-resistant intracellular enzymes (Krulwich and Guffanti 1983).

In natural environments, sodium carbonate is generally the major source of alkalinity. Its addition to the isolation media enhances the growth of alkalophilic microorganisms (Grant *et al.,* 1979). Substitutes for sodium carbonate include sodium bicarbonate, sodium sesquicarbonate, potassium carbonate, sodium borate, and sodium orthophosphate or the occasional addition of sodium hydroxide (Souza *et al.,* 1974) . The addition of Na_2CO_3 to the medium for the isolation of alkalophilic thermophiles results in brown color and cracking of the medium (Kitada *et al.,* 1987). At temperatures of 70°C, agar-based media usually lose their gel strength and exhibit water of synergisms, making them useless for isolation of thermophiles (Lin and Casida 1984).

MATERIALS AND METHODS: (Jaya Sudha *et al.*,2010)

Isolation of Alkalophiles (Alkaline Bacteria)

The sample was collected from the sheep upper collagen layer of skin which is obtained from the

1. S.A.Tannery of Erode, Tamil Nadu.

2.Naser Tanning company of Chennai, Tamil Nadu.

3. A.M.Sadick tanners of Bhavani, Tamil Nadu and

4. KKSK Leather Processor of Trichy, Tamil Nadu.

About 1.0g of skin sample was transferred to 99.0 ml sterilized normal saline in 250 ml conical flask and agitated (100 rpm) at 37° C for 1 hour on shaker.

The skin suspension was then diluted in serial up to 10^{-7} dilution. One ml of each dilution was poured into petri plates containing nutrient agar medium of pH 9. The inoculated plates were incubated at 37°C for 24 hours.

Screening of Bacterial Alkalophilies

Individual bacterial colonies were screened for proteolytic enzymes production on skim milk agar medium, containing skim milk 1.0%, peptone 0.5%, sodium chloride 5% and agar 2.5%. The pH of the medium was adjusted to 9 with 1 N HCL/ 1 N NaOH before sterilization at 120°C for 15 minute. The inoculated plates were then incubated at 37°C for 48 hours and observed for zones of clearance which indicate proteolytic activities.

Identification of The Proteolytic Isolates

The bacterial isolates with prominent zones of clearance were processed for the determination of morphology, gram characteristics, motility, citrate utilization, oxidase, urease, gelatin liquidation, catalase, VP , and indole tests, acid production from D-Glucose, D-Arabinose, D-Lactose, D-Mannitol, D-Galactose and D-Maltose. The isolates were also grown at different temperature, pH and NaCl concentration. These isolates were then identified in accordance with the methods recommended in Bergey's Manual of Determinative Bacteriology. (John G.Holt,Lippincott Williams and Wilkins 1994).

Quantitative assy for Alkaline Protease (Sai Smita *et al.*, 2012)

Preparation of Crude Enzyme Extract After incubation the Broth cultures were subjected to centrifugation at 10,000 rpm for 10 minutes at 4^{o}C using refrigerated centrifuge.

The supernatant obtained after centrifugation was used to determine the amount of the extracellular protease released into the assay medium.

Determination of Proteolytic Activity

Proteases activity was determined by a modified method. The reaction mixture containing 1 ml of 1.0% casein solution in 0.05M Glycine-NaOH buffer having pH 10 and 1 ml of given enzyme solution were incubated at 40 ^{o}C for 20 minutes and the reaction was then stopped with 3ml of 10% tri-chloroacetic acid. The mixture was allowed to stand for some time and was then centrifuged at 10,000 rpm for 10 minutres at 4 ^{o}C. 0.5 ml of the filtrate was then diluted with 4.5ml of Glycine-NaOH buffer having pH 10. The absorbance of the liberated tryosine in the filtrate was measured against a blank (non-incubated sample) at 280 nm. One proteolytic unit was defined as the amount of the enzyme that released 1µg of tyrosine under the assay conditions. Standard graph of tyrosine was preferred using 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140 and 150 mg/ml tyrosine in 50mM Glycine-NaOH buffer, pH10.

Optimization for Production of Alkline Protease (Sai Smita *Et.Al* 2012)

Optimization at different pH

The effect of pH on alkaline protease production from the isolate N-4 was determined by growing the isolate in assay medium with different alkaline pH in the range 8-12 using required concentration of 1 N NaOH.

Optimization at different time period

To study the effect of time for optimization of protease production, the isolate N-4 was inoculated in to the assay medium with pH 9 and 10 respectively. Further the organism was incubated at 37^{o}C in water bath shaker to obtain a uniform growth. The alkaline protease activity was monitored at regular time intervals of 6, 18, 24, 48 and 72 hour's duration.

Optimization at different temperature

The effect of temperature on protease production by isolate N-4 and was determined by growing in assay media at varied temperatures (4°C, 20°C, 37°C and 50°C) with the pH and time of incubation remains constant at 9 and 48 hours. The protease assay was carried out to determine the concentration of enzyme.

Optimization of Inoculum size

As production enzyme is very well correlated with concentration of inoculum provided in the assay medium, the effect of inoculums size on protease production was carried out. Varied inoculum concentration of 0.1 ml, 0.5ml, 1ml, 1.5ml, and 2 ml of the isolate was inoculated into the assay medium for protease production and the protease assay was carried out.

RESULT AND DISCUSSION

Isolation of Protease Producing Bacteria

In this study 21 bacterial strains were isolated from decaying leather samples. The samples suspension processed on skim milk containing medium. Our great concern was to isolate and identify alkalophilic enivornment, having a vital tendency to secrete extra-cellular proteolytic enzyme. Out of 21 isolates 15 exhibited vivid zones of clearance on skim milk agar medium at pH 10. The 21 isolates from different area and result were examined and tabulated such as 1. S.A.TANNERY ERODE (Table 1). 2. NASER TANNING COMPANY,CHENNAI (Table 2). 3. KKSK LEATHER PROCESSER(P) LTD, TRICHY .(Table 3) and 4. A.M.SADICK TANNERIES, BHAVANI.(Table 4).

The physical properties, Biochemical characteristics , proteolytic index and protease activity of the test organisms were recorded . It can be concluded that the isolates most likely belong to *Bacillus* sp and *Pseudomonas* sp

Protease Activity

Protease enzymes activity S-1, S-2,S-3,S-4,S-5,S-6

S.A.Tannery Erode (Fig 1)

Protease enzymes activity N-1, N-2,N-3,N-4,N-5,N-6

Naser Tanning Company Chennai (Fig 2)

Protease enzymes activity K-1, K-2, K-3, K-4, K-5

Kksk Leather Processer(P)Ltd Trichy (Fig 3)

Protease enzymes activity S-1, S-2,S-3,S-4,S-5,S-6

A.M.Sadick Tanneries Bhavani (Fig 4)

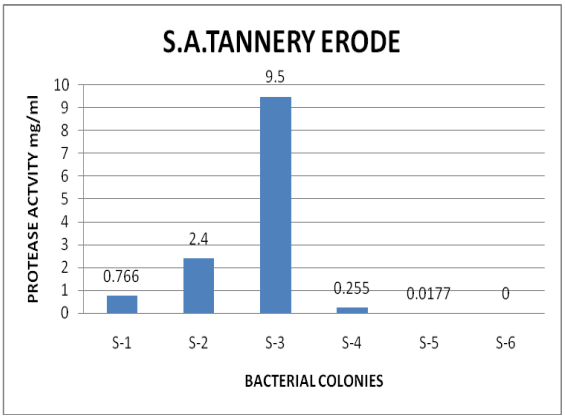

Fig:1S.A.Tannery Erode

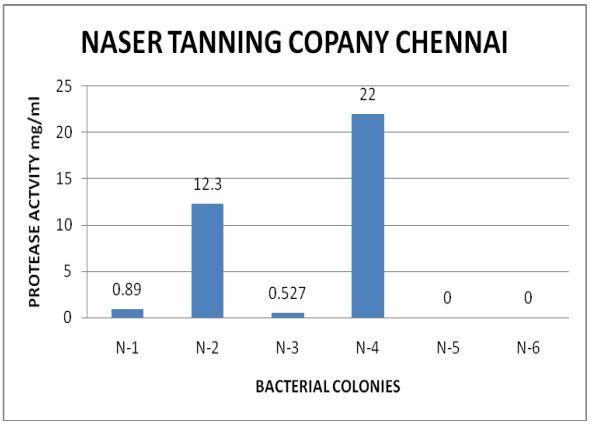

Fig: 2 Naser Tanning Company Chennai

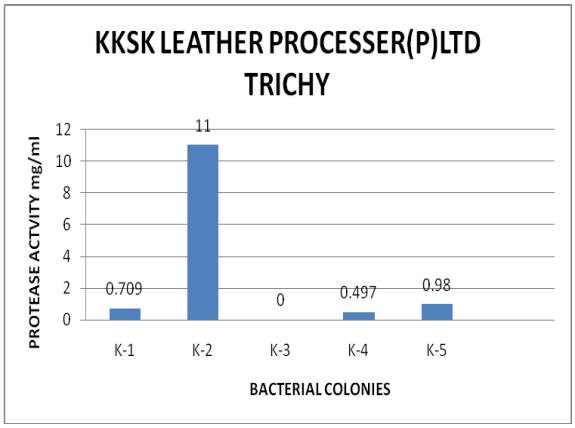

Fig: 3 Kksk Leather Processr (P) Ltd, Trichy

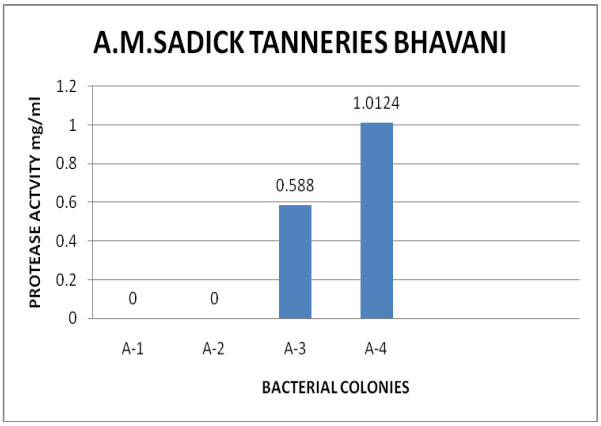

Fig: 4 A.M.Sadick Tanneries, Bhavani

Table1: Bacterial Colonies Isolated from Leather Sample of S.A.Tannery, Erode.

S.N.	TEST	S-1	S-2	S-3	S-4	S-5	S-6
1	SHAPE	ROD	ROD	**ROD**	ROD	ROD	ROD
2	MOTILTIY	+	+	+	+	+	+
3	GRAM STAINING	+	+	+	-	+	+
4	SPORE STAINING	+	+	+	-	+	+
5	INDOLE TEST	-	-	-	+	-	-
6	METHYLE RED TEST	+	-	+	-	-	-
7	V.P.TEST	+	+	+	-	+	+
8	CITRATE UTILIZATION TEST	+	-	-	-	-	-
9	NITRATE REDUCTION	+	+	+	-	+	+

10	GELATINE HYDROLYSIS TEST	+	+	+	+	+	+
11	CATALASE TEST	+	+	+	+	+	+
12	STARCH HYDROLYSIS TEST	+	+	+	-	+	+
13	CARBOHYDRATE FERMENTATION TEST						
1	GLUCOSE	+	+	+	+	+	+
2	LACTOSE	-	+	-	-	+	-
3	MALTOSE	+	-	+	-	-	+
4	FRUCTOSE	+	+	+	-	+	+
14	PROTEASE ACTIVITY TEST	0.766 mg/ml	2.4mg/ml	**9.5mg/ml**	0.255 mg/ml	0.0177mg/ml	NIL

Table 2 :Bacterial Colonies Isolated from Leather Sample of Naser Tanning Company, Chennai.

S.N.	TEST	N-1	N-2	N-3	N-4	N-5	N-6
1	SHAPE	ROD	**ROD**	ROD	**ROD**	ROD	ROD
2	MOTILTIY	+	+	+	+	+	+
3	GRAM STAINING	+	+	+	+	-	-
4	SPORE STAINING	+	+	+	+	-	-
5	INDOLE TEST	-	-	-	-	+	+
6	METHYLE RED TEST	-	-	-	+	-	-
7	V.P.TEST	+	+	+	+	-	-
8	CITRATE UTILIZATION TEST	+	+	+	+	+	+
9	NITRATE REDUCTION	+	+	+	+	-	-
10	GELATINE HYDROLYSIS TEST	+	+	+	+	+	+
11	CATALASE TEST	+	+	+	+	+	+
12	STARCH HYDROLYSIS TEST	+	+	+	+	+	+
13	CARBOHYDRATE FERMENTATION TEST						
1	GLUCOSE	+	+	+	+	+	+

2	LACTOSE	+	-	-	-	-	-
3	MALTOSE	-	+	+	+	-	-
4	FRUCTOSE	+	+	+	+	-	-
14	PROTEASE ACTIVITY TEST	0.890 mg/ml	**12.3 mg/ml**	0.527 mg/ml	**22mg/ml**	NIL	NIL

Table 3 Bacterial Colonies Isolated from Leather Sample of Kksk Leather Processer (P) Ltd, Trichy.

S.NO	TEST	K-1	K-2	K-3	K-4	K-5
1	SHAPE	ROD	**ROD**	ROD	ROD	ROD
2	MOTILTIY	+	+	+	+	+
3	GRAM STAINING	-	+	+	+	+
4	SPORE STAINING	-	+	+	+	+
5	INDOLE TEST	+	-	-	-	-
6	METHYLE RED TEST	-	+	+	+	-
7	V.P.TEST	-	+	+	+	+
8	CITRATE UTILIZATION TEST	+	-	+	+	+
9	NITRATE REDUCTION	-	+	+	+	+
10	GELATINE HYDROLYSIS TEST	+	+	+	+	+
11	CATALASE TEST	+	+	+	+	+
12	STARCH HYDROLYSIS TEST	-	+	+	+	+
13	CARBOHYDRATE FERMENTATION TEST					
1	GLUCOSE	+	+	+	+	+
2	LACTOSE	-	-	-	-	-
3	MALTOSE	-	+	+	+	-
4	FRUCTOSE	-	-	+	+	+
14	PROTEASE ACTIVITY TEST	0.709 mg/ml	**11mg/ml**	NIL	0.497 mg/ml	0.98 mg/ml

Table 4 Bacterial Colonies Isolated from Leather Sample of A.M.Sadick Tanneries, Bhavani.

S.NO	TEST	A-1	A-2	A-3	A-4
1	SHAPE	ROD	ROD	ROD	ROD
2	MOTILTIY	+	+	+	+
3	GRAM STAINING	-	-	+	+
4	SPORE STAINING	-	-	+	+
5	INDOLE TEST	+	+	-	-
6	METHYLE RED TEST	-	-	-	-
7	V.P.TEST	-	+	+	+
8	CITRATE UTILIZATION TEST	+	+	+	+
9	NITRATE REDUCTION	-	-	+	+
10	GELATINE HYDROLYSIS TEST	+	+	+	+
11	CATALASE TEST	+	+	+	+
12	STARCH HYDROLYSIS TEST	-	-	+	+
13	CARBOHYDRATE FERMENTATION TEST				
1	GLUCOSE	+	+	+	+
2	LACTOSE	-	+	-	+
3	MALTOSE	-	+	-	-
4	FRUCTOSE	-	+	+	+
14	PROTEASE ACTIVITY TEST	NIL	NIL	0.588 mg/ml	1.0124 mg/ml

Effect of Ph on protease Enzyme production

From the pH optimization study shown in (Fig 5), it was clearly observed that N-4 was able to produce 22mg/ml at pH 10. It was observed that there is a gradual increase in protease production from 8-10 pH, subsequently the production capacity was showing a downward trend after pH-9.The enzyme synthesis was increased with increase of medium pH towards alkaline range from neutrality. (Prakasham, *et al.*,2006) and alkaline protease production strongly depends on the extra-cellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell growth and product production (Ellaiah *et al.*, 2002).

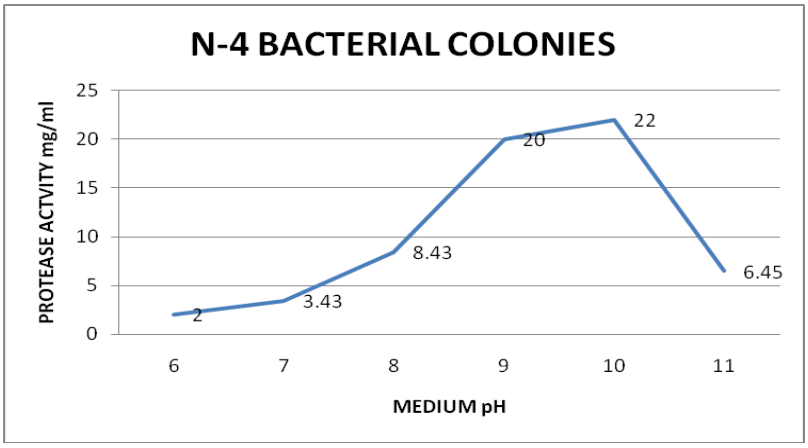

Fig:-5 Optimization of Medium pH

Effect of Time on protease production

Result of this study showed that protease production increased with incubation time (Fig 6). Enzyme synthesis is related to cell growth and therefore there is a co-relation between incubation period and enzyme production (Kaur *et al.*, 1998). During the early growth phase of 6 to 18 hours, protease production proceeded at a slower rate after which it increased sharply reaching a maximum value at 48 to 60 hours. This decline might be due to cessation of enzyme synthesis together with auto proteolysis.

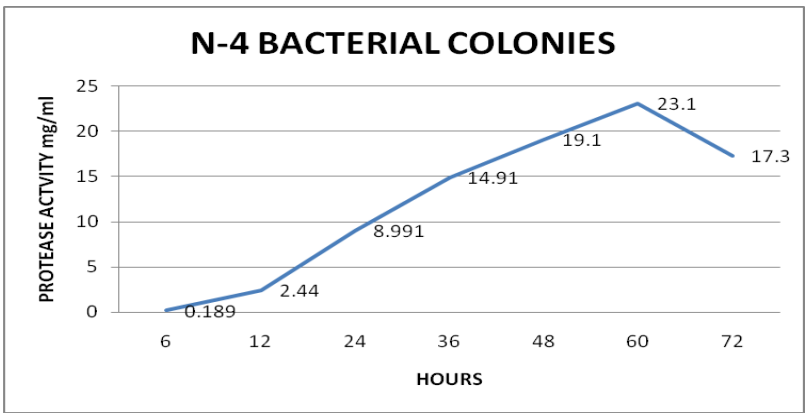

Fig:-6 Optimization of Time for Protease production

Effect of Growth Temperature on protease production

The effect of temperature on alkaline protease production revealed that maximum yield was obtained at 37°C (22.34 mg/ml) (Fig 7). A decrease in enzyme yield was observed with further increase in temperature. which is a common phenomenon in many fermentation process. The temperature associated decreases in yield may have been due to the fact that denaturation or degradation of the proteolytic enzyme by autolysis in response to elevated temperatures caused alkaline protease activity to decrease (Joo 2001).

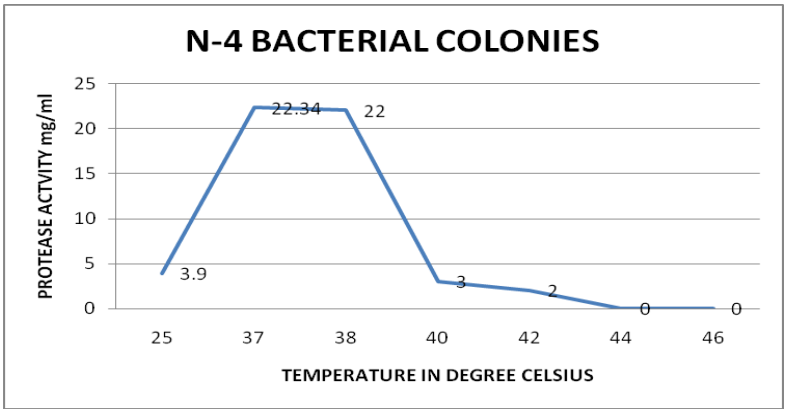

Fig: 7 optimization of Temperature

Effect of inoculum Size on protease production

Result from the present study showed that optimum inoculum size of the bacterial isolate for protease production was 1ml from overnight culture broth (Fig 8). The less protease production in small inoculum size of 0.1, 0.2 and 0.8 ml may be due to insufficient number of bacteria, which would have lead to reduced amount of secreted protease and the decrease even through luxurious growth was observed in higher inoculum size of 1.5 and 2 ml. A similar observation has been reported by some authors (El-Safey and Abdul-Raouf, 1995).

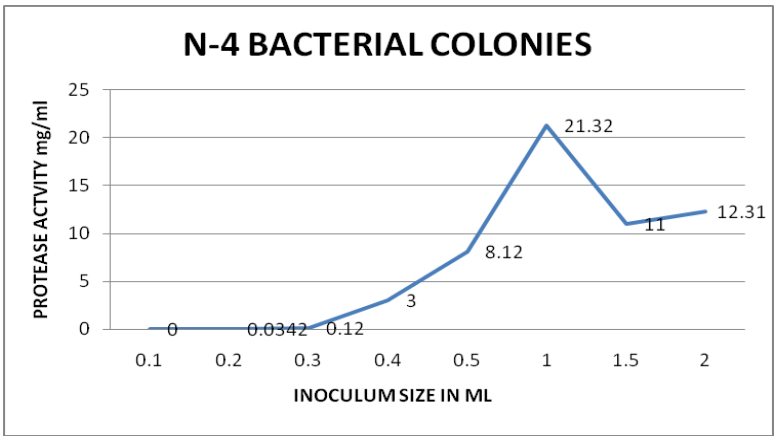

Fig 8 Optimization of Inoculum size for protease production

CONCLUSION

In the present investigation protease producing bacteria from leather processing industry were screened. Only few bacteria grown on alkaline containing medium, that produce alkaline protease were discovered. Among the 21 colonies screened some of them produced the highest protease in submerged culture medium. Based on physical and biochemical properties of the isolates screened most likely the organisms belong to *Bacillus* sp. The use of

protease would also make it possible to reduce the use of harmful chemicals and wastes in the environment because these chemicals can be replaced by protease which are more efficient.

These properties indicate the potential use of the enzymes in detergent and leather industries. Further investigation is needed to identify the natural selection medium, pH, temperature, agitation, aeration, submerged fermentation and commercial application values

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