Int.J.Curr.Microbiol.App.Sci (2016) *5*(5): 767-787

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 5 (2016) pp. 767-787**
Journal homepage: http://www.ijcmas.com

Original Research Article http://dx.doi.org/10.20546/ijcmas.2016.505.078

Screening and Characterization of Alkaline Protease Producing Bacillus Strain B-4 *Bacillus flexus* and Study of its Potential for Alkaline Protease Production

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Keywords

Alkaline protease, Bacillus, Relative enzyme activity, optimization.

Article Info

Accepted:
20 April 2016
Available Online:
10 May 2016

A B S T R A C T

Microbial proteases account for approximately 40% of the total worldwide enzyme sales and most of the commercial proteases are of bacterial origin. So planned to screen out bacterial cultures from various sources producing significant amount of alkaline proteases. Samples collected were serially diluted and plated on skim milk agar plates and incubated at 37 ºC for 3-4 days to allow the colonies to grow. Zone of clearance of casein surrounding the colony has provided a measure of their Proteolytic activity. The isolates showing zone of casein hydrolysis on milk agar plates were studied for their relative enzyme activity (REA) on skim milk agar plate. To compare the caseinolytic activity of enzyme produced, the isolates were inoculated in production medium and put on an environmental shaker at 100 rpm at 37ºC for 168 hours and checked for enzyme activity at interval of 24 hours. Selected isolateB-4 was studied for cultural, morphological, biochemical characteristics and identified by 16S rRNA sequencing and also employed for optimization of cultural conditions and components of production medium. We also employed the crude enzyme for its efficiency of gelatin hydrolysis and silver extraction from used X-ray films.

Introduction

Enzymes are the catalytic cornerstones of metabolic activities of living being and catalyze most of the reactions in living organisms. Microbes secrete proteases to hydrolyze the peptide bonds in proteins and therefore break the proteins down into their constituent monomers. Bacterial alkaline proteases are characterized by their high activity at alkaline pH and their broad substrate specificity. Their optimal temperature is around 60°C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry and other commercial fields. Appropriate nutritional environments must be created and maintained to ensure optimized yields and product quality. Culture media optimization is a critical step in fermentation process development and often continues throughout the production life of the fermentation product. In this study

we tried to screen out alkaline protease producing bacteria and optimization of production medium for alkaline protease production by the best isolates. Crude enzyme was employed for its efficiency of silver recovery of used X-ray films

Materials and Methods

Source, Samples and Medium for Isolation, Preservation and Screening

Various samples of soil and water were collected from different regions of Gujarat which included; soil from Umreth chicken field, waste water sample, pond water sample from pij, Umreth cattle field, kitchen waste water sample, and company waste soil sample. Soil samples were aseptically collected from top soil surface. One gram of soil sample and 1 ml of water sample of each site were serially diluted using sterile distilled water and 100µl aliquots were placed on skim milk agar plates and incubated at 37ºC for 2-3 days to allow the colonies to grow. The well isolated colonies were marked and colony characters and morphological characters were noted at the interval of 24 h, diameter of zone of clearance of casein was also measured which provided a measure of their Proteolytic activity. Each isolate was transferred on milk agar plates two to three times to get confirm isolated pure cultures. Purity of cultures was confirmed by Gram staining (Bergey David *et al.,* 1994). Cultures were preserved at 4ºC on casein agar slants. Sub culturing was carried out every 30- day's interval.

Selection of potent isolates and study of their cultural characters

Fresh culture isolates were taken and small drop was put in the middle of skim milk agar plate and incubated at 37ºC for 5-6 days and at interval of 24 h., zone of casein hydrolysis and diameter of growth were measured and relative enzyme activity (REA) was calculated (Jain R. *et al.,* 2009). (REA =Diameter of zone of casein hydrolysis/ Diameter of colony in mm.) Based on REA, organisms were categorized into three groups showing excellent (REA>5), good (REA>2.0 to, 5.0) and poor (REA<2) producer of protease.

Study of Cultural Characteristics and Morphology

The isolates showing zone of casein hydrolysis on milk agar plates, were marked, labeled and observed to note down their colony characters. Colony characters taken in consideration were, colony size, shape, elevation, margins, opacity, pigmentation, reverse side pigment, pigment solubility, texture etc. and also studied morphological characteristics along with their spore arrangement by performing Grams Staining (Bergey *et al.,* 1994).

Study of Biochemical Activity

On the basis of zone of casein hydrolysis, potent isolates were selected for further study. The isolates were characterized for their biochemical activity. The biochemical tests carried out for the isolates were: indole production test, methyl red test, Voges proskauer test , citrate utilization test, nitrate reduction test, ammonia production test, catalase test, urea utilization test, gelatin hydrolysis test, hydrolysis of starch, H2S production test, dehydrogenase test. Growth pattern in broth, Carbohydrate utilization test.

16 S rRNA Sequencing of Potent Isolates

Isolates preserved on Nutrient casein agar slants were sent to Gujarat State

Biotechnology Mission (GSBTM, Gandhinagar) for 16SrRNA sequencing and the BLAST match was used for confirm identification of the isolate B4.The sequences obtained from GSBTM Gandhinagar were analyzed at NCBI server (www.ncbi.nim.nih.gov) using BLAST tool and have been submitted to GeneBank. The phylogenetic trees of the isolate was constructed with MEGA version 6.0 using the neighbor joining method (Tamura *et al.*, 2007).

Microorganisms and Inoculum preparation

The potent protease producing *Bacillus flexus* B4 were selected for media optimization for their protease production. 48h pure growth of *Bacillus flexus* B4 was obtained on milk agar medium (pH 8.5).The fresh culture from plate was transferred in to 25 ml distilled water and mixed well and the turbidity was adjusted to make its OD 0.75 at 670nm against distilled water as blank.

Measure of Protease Production

To compare the caseinolytic activity of enzyme produced by both the isolates, both the isolates were inoculated in production medium consisting of glucose150mg, K2HPO4 20mg, KH2PO4 20mg, MgSO4 10mg, CaCl2 10mg, casein 200mg, NaNO3 100mg, 100ml distilled water, pH-8.5(Rao and Narasu 2007) and put on an environmental shaker at 100 rpm at 37 ºC for 144 h and checked for enzyme activity at interval of 24h.

The supernatant was collected after centrifugation at 10,000 rpm for 15 minutes and used as crude enzyme source Proteolytic activity in the supernatant was determined by using spectrophotometer method, given by Anson - Hagihara (1958) with minor modifications.

Initial production medium, enzyme assay and measurement of total protein

The initial enzyme production by *Bacillus flexus* B4 to check incubation period

Bacillus flexus B4 was inoculated in 250ml Erlenmeyer flasks containing 100 ml of production medium, consisting of glucose150mg, K2HPO4 20mg%, KH2PO4 20mg%, MgSO4 10mg%, CaCl2 10mg%, casein 200mg%, NaNO3 100mg%, (pH 8.5) (Rao and Narasu, 2007) and put on an environmental shaker at 100 rpm at 37ºC for 168h and checked for enzyme activity at interval of 24h. The supernatant was collected after centrifugation at 10,000 rpm for 15 minutes and used as crude enzyme source.

Enzyme Assay and Measurement of Total Protein

Proteolytic activity in the supernatant was determined by using spectrophotometer method, given by Anson-Hagihara (1958) with minor modification (Takami *et al.*, 1989). Activity of enzyme was measured in terms of unit. (µg/ml/min) One unit of enzyme is defined as the quantity of enzyme required to release 1µg of tyrosine per minute, under the standard assay conditions (Hameed *et al.*, 1999). Protein content was measured by Lowry's method with BSA as a standard protein (Lowry *et al.*, 1951).

Effect of Incubation Temperature on Protease Production

Effect of temperature on the production of extracellular protease production was analyzed by inoculating the isolate in various 250ml Erlenmeyer flasks containing 100 ml of production medium and then incubated at different temperatures (30, 37, 40, 45, 55ºC) on environmental shaker at

100 rpm for 24h. After incubation period, from each flask, protease production was checked in terms of protease activity and results were analyzed for optimum temperature for maximum protease production by the isolate.

Effect of initial pH of the Medium

Effect of Initial pH of the production medium on production of extracellular protease was studied by assaying the enzyme after 24h of incubation at 37ºC by adjusting the initial pH of the production medium to different pH values ranging from 7.0 to 11 using appropriate buffers. Tris HCl buffer (pH 6.0 -8.0), Glycine NaOH buffer (pH 8.0-11).

Change in pH of the culture medium during the fermentation process was checked by inoculating 250ml Erlenmeyer flask containing 100 ml of production medium with initial pH values 9.5(as found optimum) and then incubated on an environmental shaker at 100 rpm at 37ºC for 168h. pH change occurring in culture medium along the progress of fermentation was recorded at the interval of 24h.

Determination of Optimum pH for Enzyme Activity

The optimum pH for enzyme activity was determined by assaying the activity of purified enzyme at various pH values from 6 to 12. Substrates (1% casein) were prepared in different buffers 50 mM: Sodium acetate buffer pH 5.0; Tris-acetate buffer pH 6.0; Tris-acetate buffer pH 7.0; Tris-HCl buffer pH 7.2, 8.0 and 9.0; Glycine-NaOH buffer pH 10.0 and 11.0. The Caseinolytic activity was determined using these substrates in the assay system at70°C for 20 min. Maximum activity yielding pH was considered as optimum pH for activity

Effect of Inoculum Size on Protease Production

The effect of Inoculum size of the culture B.flexus B4 was carried out by growing the different volumes of the inoculum 1.0 ml, 2.0 ml, 3.0 ml, 4.0 ml and 5.0 ml (OD 0.75 at 660nm) of isolate for 24h at 37ºC,on an environmental shaker at 100pm. After incubation period, protease production was checked in terms of protease activity and results were analyzed for optimum inoculum size of the culture for maximum protease production by the isolate.

Effect of Carbon Sources on Protease Production

Production medium was prepared with different carbon sources like, mixture of glucose and casein, starch, sucrose, sodium citrate, lactose at 150mg% w/v and glycerol 0.5% v/v. Inoculum of the culture B.flexus B4 was inoculated in production media with different carbon sources and then incubated for 24h at 37ºC temperature on environmental shaker at 100 rpm. Best carbon source was determined for maximum protease production.

Effect of Nitrogen Sources on Protease Production

Different organic and inorganic nitrogen sources like, NaNO3, peptone, yeast extract, aspargine, ammonium sulphate, lysine, urea and mixture of peptone and casein at 150 mg% w/v were incorporated in production medium and Inoculated with *B. flexus* B4, and incubated for 24h at 37ºC temperature on environmental shaker at 100 rpm. After incubation period, protease production was checked and results were analyzed for best nitrogen source for maximum protease production.

Enzymatic hydrolysis of gelatin and bioprocessing of X-ray film for silver recovery:

Used X-ray film were washed with distilled water and wiped with cotton impergnated with ethanol. The washed film was dried in an oven at 40˚C for 30 min. One X-ray film (cut into 2X2 cm pieces) was then incubated for 1h at 70˚C with 20% (68.37 units/ml) of crude protease (Nakiboglu *et al.,* 2003) in different petri plates in glycine NaOH buffer of ph 9.5 such that the film is completely immersed in the solution (total volume150 ml). The results were recorded at an interval of 10 min. Control was prepared using buffer without enzyme addition. Removal of gelatin from the film was also measured by Lowry's method (1951) for quantifying the gelatin content in the stripped solution with bovine serum albumin (BSA) as the standard. Furthermore, turbidity of the removed solution was also measured at 670 nm. Presence of silver ions was detected by addition of potassium chromate and NaCl in the stripped solution by precipitation of silver salts.

Result and Discussion

Screening of Protease Producing Microorganisms

Screening of alkaline protease producing bacteria from various sources (Table: 2) was carried out using alkaline skim milk agar medium. Out of forty seven total isolates, four potent Actinomycetes and four Bacilli were selected showing zone of casein hydrolysis surrounding their colonies. All were alkaliphilic and having diverse morphological characters. (Figure:1)

As shown in above photographs, on the basis of visual observations and measuring casein hydrolysis zone size, colonies were marked and colony characteristics were noted and Gram staining of all eight isolates were performed and presented (Table:2).

Comparative REA (relative enzyme activity) of Isolates

On the basis of morphology and cultural characteristics, it was confirmed that the isolates A1, A2, A3 and A4 were protease producing Actinomycetes and isolates B1, B2, B3 and B4 were Bacilli and both were producing good amount of alkaline protease on solid media. This was confirmed by performing REA. Most of them were having REA more than 2.0 except A4. Figure 2 A and B and Figure :3. Highest REA 3.42 was observed for Actinomycete strain A1.Similar reports were made by Richa Jain (2009) for various Streptomyces species like, Str. exfoliates CFS1068(REA=10), Str. somaliensis GS 1242 (REA=8.8), Str. sampsonii GS 1242 (REA=9.6) by the similar method.

Selection of Potent Protease Producing Isolates

Most of the isolates were having REA more than 2.0 except A4.So we counter checked them for their protease production capacity in production medium suggested by Rao and Narasu, 2007. When production profile of all the isolates were compared, among baciillli, B4 was found producing maximum amount of protease within 96h (59.18 units/ml) Figure: 4.

Identification of Potent Protease Producing Isolate B4

As we have decided to work with Bacilli, most explored bacteria for enzyme production, and producing enzyme faster within 24h in larger quantities, we selected B4 for further studies and identification for which we relied upon: Cultural and morphological characteristics, Biochemical characters and Molecular identification by 16S r RNA sequencing

Cultural Characteristics of B4

The cultural characteristics, morphological characteristics and spore nature of the isolate B4 are presented in Table. 3 and photographs of the growth characteristics and gram staining are presented in Figure: 5 a and b.

Study of Biochemical Activity of Potent Isolate B4

Results of biochemical tests carried out for isolate B4 are presented in Table :4. which are very much similar to that of *Bacillus flexus*.

16 S rRNA sequencing and phylograms of isolate B4

A molecular approach was necessary to support unambiguous identification of isolates at species level. Molecular systematic, which includes both classification and identification, has its origin in the early nucleic acid hybridization studies, but has achieved a new status following the introduction of nucleic acid sequencing techniques (O'Donnell *et al.,* 1993).

Significance of phylogenetic studies based on16S rRNA sequences is increasing in the systematics of bacteria and actinomycetes (Yokota, 1997). Here, 16S rRNA sequencing of potent isolate B4 is presented in Table (Table 3.4).

Depiction of the phylogenetic tree derived from 16SrRNA sequences of isolate B4 is presented in Figure :6 which showed that the sequence exhibited a high level of homology with *Bacillus flexus*. Based on morphological, biochemical and molecular data, it was confirmed that isolate represented a species of Bacillus and designated as. *Bacillus flexus*. B4.

Protease production and optimization of certain parameters

The protease production profile of B4 to determine incubation period of fermentation

The study of enzyme production is presented in Figure: 7 in which it was indicated that incubation period for best production was 24h.where maximum protease activity (60.19 units/ml) was noticed. So throughout the study we considered 24h as incubation period for the fermentation. Similar kinds of results were also reported for B. subtilis AKRS3 Krishnan Ravishankar *et al.,* 2012.

Effect of Temperature on Protease Production by Isolate B4

It is known that temperature is one of the most critical parameters that have to be controlled in bioprocess (Chi and Zhao, 2003). The growth and enzyme production are greatly influenced by incubation temperature. The effects of different incubation temperatures on protease production were evaluated and it was found that 37ºC was the most favorable temperature for protease production by the isolate B4. (Figure: 8).Below 35ºC and above 45ºC, protease production was negligible. Similar reports were recorded for B. subtilis AKRS3 Krishnan Ravishankar *et al.,* 2012, for *Bacillus licheniformis* by B.K.L. Lakshmi *et al.,* 2014, for Bacillus clausii I-52 by Han-SeungJoo, *et al.,* 2006, for Bacillus cereus SV1by Laila Manni *et al.,* 2010.

Effect of Initial pH of medium on protease production by isolate B4

pH of the production medium greatly affects enzyme production. (Kumar and Takagi 1999). Culture pH strongly affects many enzymatic processes and transport of several

species across the cell membrane. Variation in pH alters acid-base equilibria and fluxes of various nutrients, inducers and growth factors between the abiotic and biotic phase (Moon & Parulekar 1991). The influence of pH on cellular activity is determined by the sensitivity of the individual enzymes to changes in pH. Enzymes are normally active only within a certain pH interval and the total enzyme activity of the cell is therefore a complex function of the environmental pH.

Table.1 Composition of Skim Milk/ Case in Agar Medium

Peptone	Meat extract	NaCl	Agar	sterilized skim milk/ casein	pH
0.5 gm%	0.3 gm %	0.5 gm%	3.0 gm %	10% v/v or 1.0gm%	8.5

Table.2 Cultural and Morphological Characters of Protease Producing Isolates

Isolates	Sample source	Colony characters	Gram reaction
A1	Chicken field, soil sample, Umreth	Small, round, even, slightly raised, opaque, rough, white, orange pigment on aging	Gram +ve spore forming filamentous
A2	Waste water sample, Nadiad	Big, round, uneven, slightly raised, rough, opaque, blackish white in color	Gram +ve filamentous
A3	Pond water sample, Pij	Small, round, uneven, concave, rough, opaque, white	Gram +ve filamentous
A4	Soil sample, Fatehpura	Big, round, even, rough, raised, opaque, white	Gram +ve filamentous
B1	Cattle field, soil sample, Umreth	Big, round, uneven, slightly raised, smooth, opaque, light yellow	Gram +ve big rod singly and in chain
B2	Cattle field, soil sample, Umreth	Big, round, uneven, slightly raised, smooth, opaque, light yellow	Gram +ve rod mostly in single
B3	Kitchen waste sample, Nadiad	Big, round, uneven, raised, smooth, opaque, light orange	Gram+ve rod in bunches
B4	Soil sample, Nadiad	Big, round, uneven, slightly raised, smooth, opaque, light orange	Gram+ve rod in chain

Table.3 Cultural and Morphological Characteristics of *Bacillus* Sp B4

Isolate	Size	Shape	Margin	Texture	Elevation	Opacity	Colony Color	Morphology by Gram staining
B4	Big	Round	Uneven	Rough	Slightly raised	Opaque	Light orange	Gram positive, rod shaped, arranged in chains and singly with spore formation (Size using micrometry): 9.18µm x 1.96 µm).

Table.4 Biochemical Activity of isolate B4

No.	Test\ Org.	B4
1	M.R. test	Negative
2	V.P. test	Negative
3	Nitrate reduction test	Positive
4	Gelatinase test	Positive
5	Catalse test	Positive
6	Indole production test	Negative
7	6.5% NaCL	Positive
8	H2S production test	Negative
9	Citrate utilization test	Positive
10	Urea broth (Urease test)	Positive
11	Carbohydrate utilization test 1) Glucose 2) Arabinose 3) Xylose 4) Mannitol	 Positive Negative Positive Negative
12	N.broth (Growth pattern)	Uniform growth
13	Amylase test	Positive

Table.5 16 S rRNA Sequencing of B4 (GSBTM, Gandhinagar)

Sr. No.	Sample ID	BAB ID	*Organisms name*	Total score	Query cov	E value	% Identity	Sequence
1	B4	BAB 5672	*Bacillus flexus*	1310	99%	0	99%	>BAB 5672
ACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCTAACGCA TTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTG GAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACTCTAGAGATAGAG CGTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT CCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACC GGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTAC AAAGGGCTGCAAGACCGCGAGGTCAAGCCAATCCCATAAAACCATTCTCAGTTCGGATTGTAGGCTGCAAACTCGC CTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC GCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGCGGTAACCTTTTATGGAGCCAGCCGCCTAAGGTGG GACAGATGATTGGGGTGAAGTCGTAACAAGGTAA							

Table.6 Summary of Optimized Conditions and Medium Components

Conditions for protease production	**Optimized level**
Temperature	37 ºC
Initial pH	9.5
Incubation period	24h
Agitation	100 rpm
Inoculum size	3.0 ml(O.D. 0.75 at 660 nm)
Medium Component	Optimized level
Carbon source	Casein + Glucose 0.15gm%
Nitrogen source	casein+Peptone 0.15gm%

Table.7 Components of Optimized Production Medium in 100ml of Distilled Water

Casein	**Peptone**	**K_2HPO_4**	**KH_2PO_4**	**$MgSO_4$**	**$CaCl_2$**	**pH**
200mg	150 mg	20mg	20mg	10mg	50mg	9.5

Fig.1 Skim Milk Agar Medium Showing Colonies of Protease Producers from Different Samples

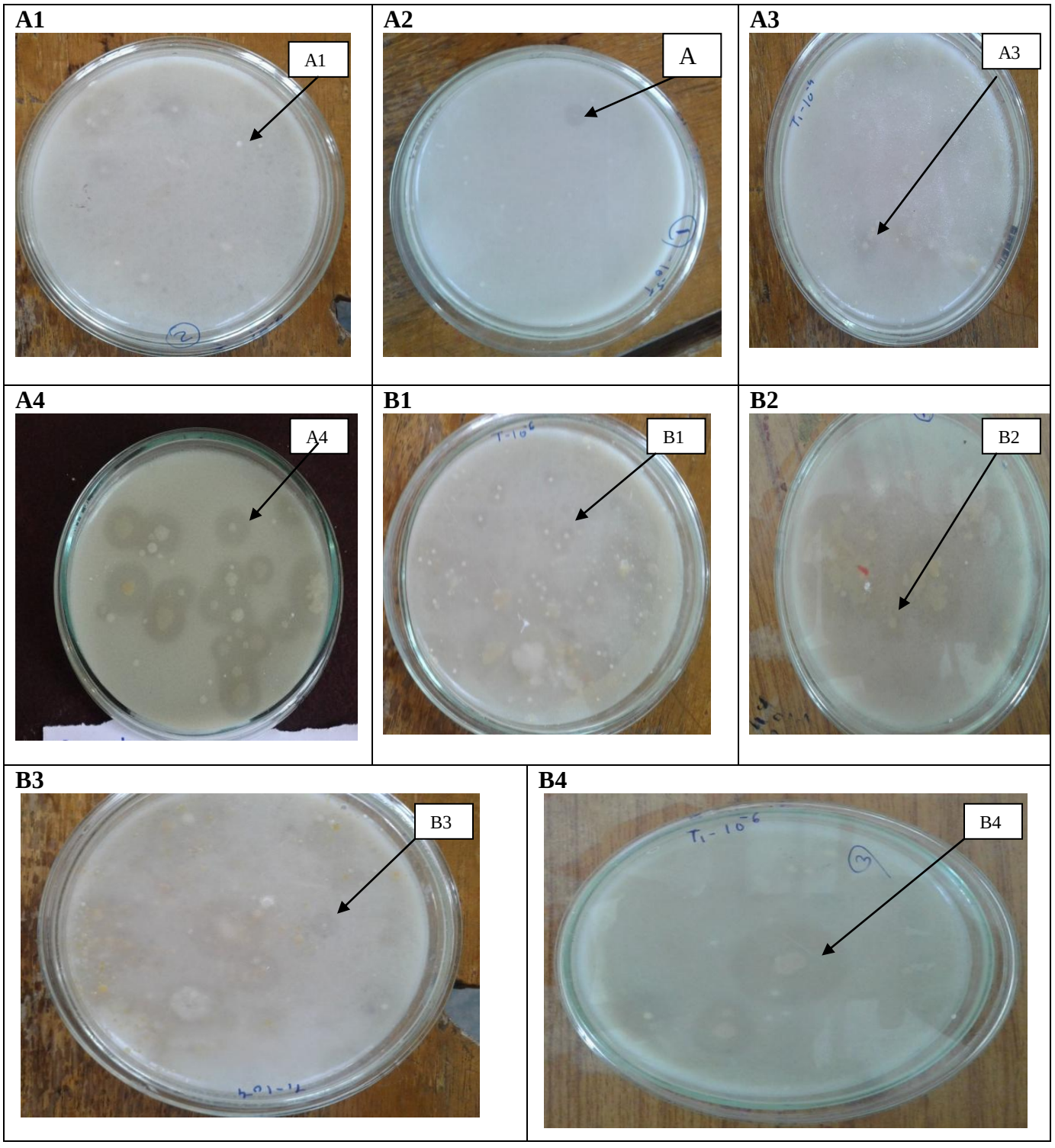


Fig.2 A: Spot Test of Protease Producing Bacilli.

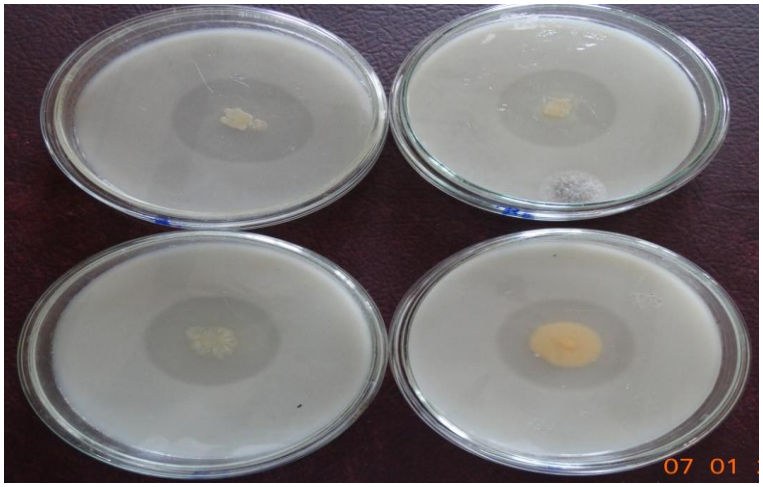


Fig.2 B: Spot Test of Protease Producing Actinomycetes.

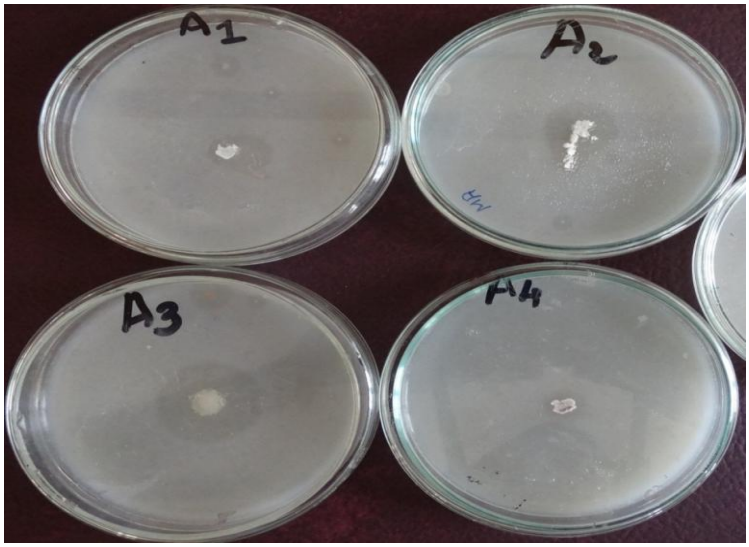


Fig.3 Relative Activities of Isolates

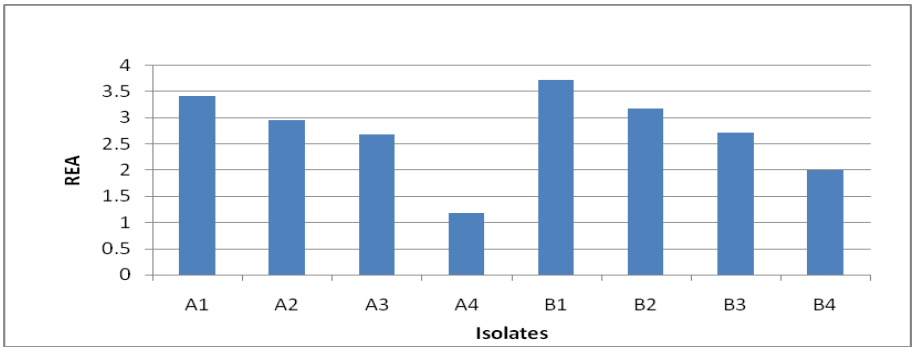


Fig.4 Protease Production Profile of Isolates at their Respective Incubation Time

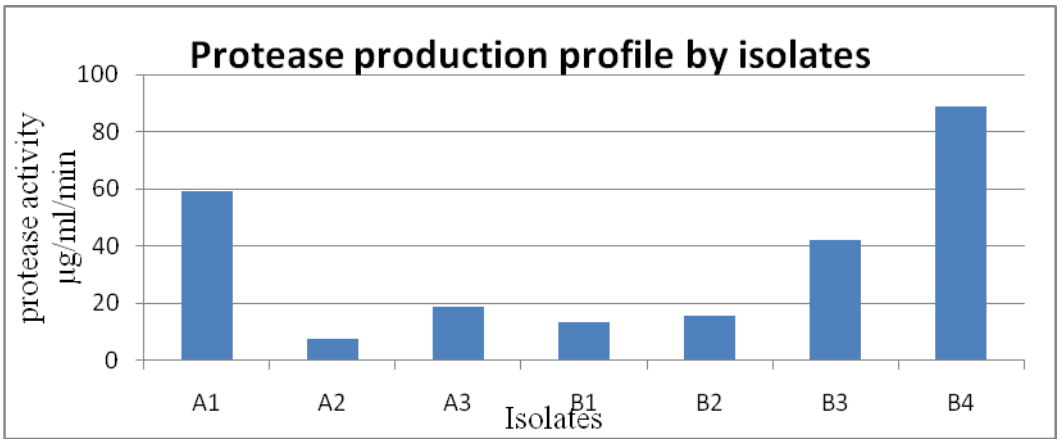


Fig.5 a. Growth Characteristics of Isolate B4 on Skim Milk Agar Plate.

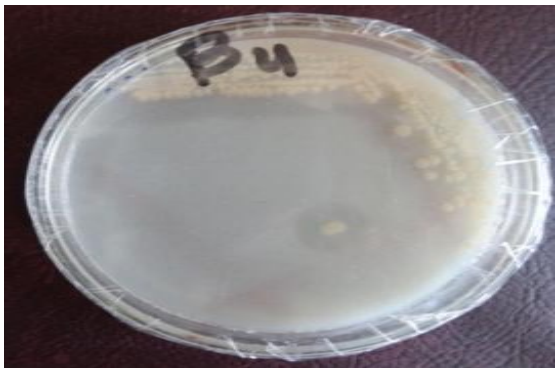


Fig.5 b. Morphology by Gram's Staining of Isolate B4.

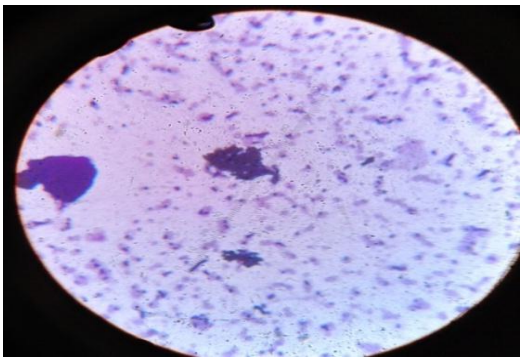


Fig.6 Phylogram of Strainb4 *Bacillus flexus*

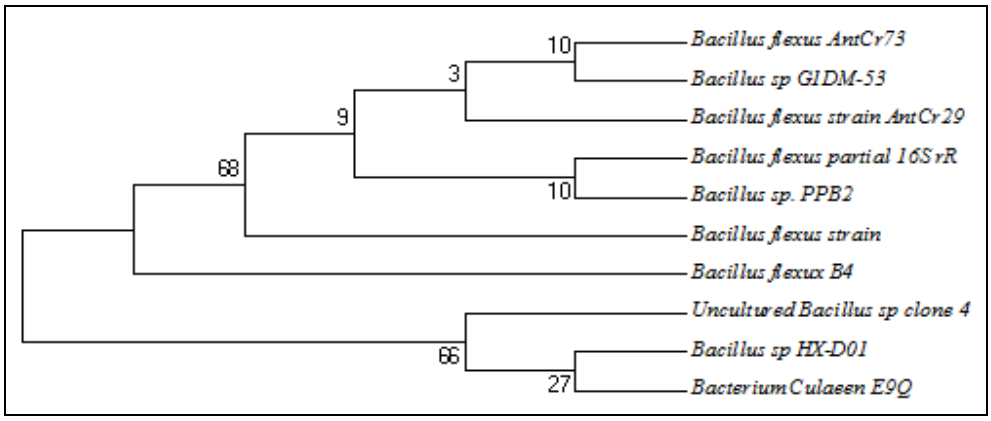


Fig.7 Protease Production Profile of Isolate B4.

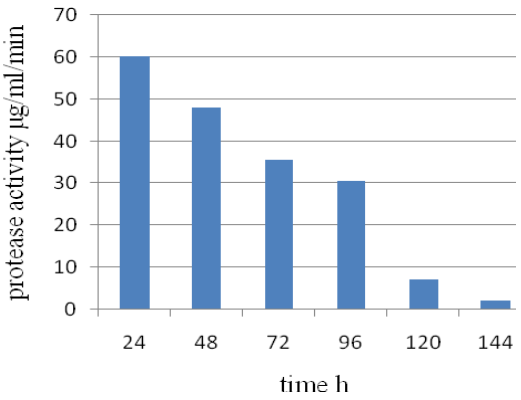


Fig.8 Effect of Temperature on Protease Production by Isolate B4.

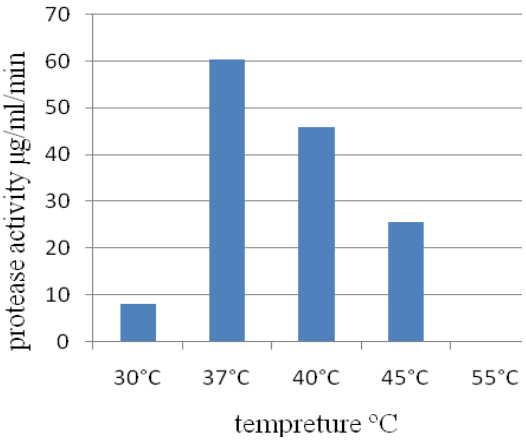


Fig.9 Effect of Medium Ph on Protease Production

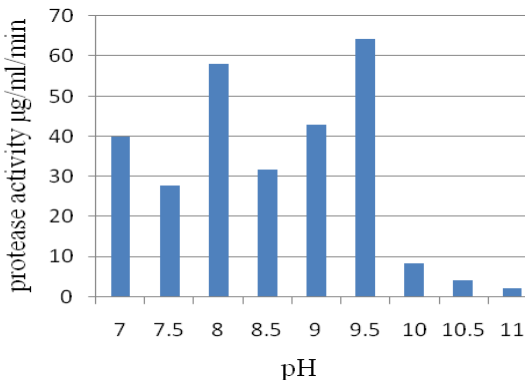


Fig.10 Study of Optimum pH for Caseinolytic Activity of Crude Protease of B4

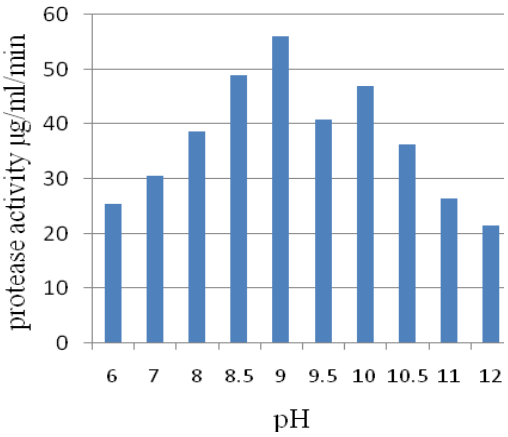


Fig.11 Effect of Inoculum Volume on Protease Production by B4

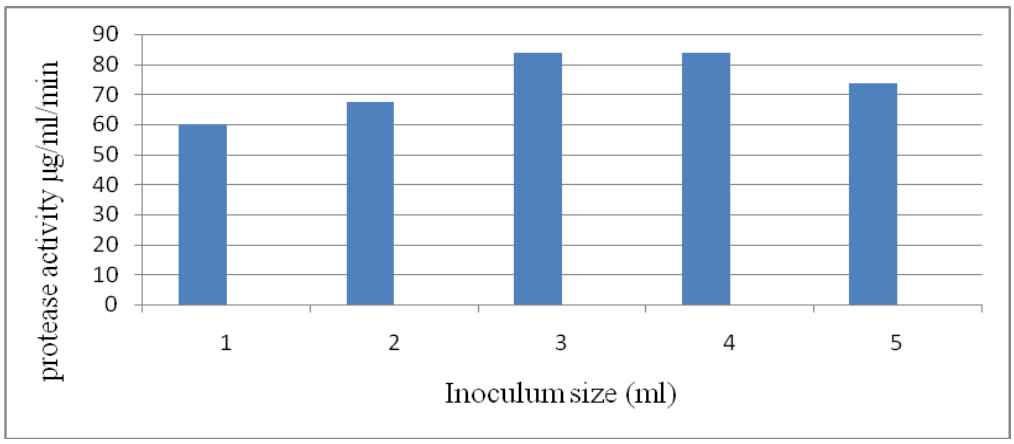


Fig.12 Effect of Carbon Sources on Protease Production By B4

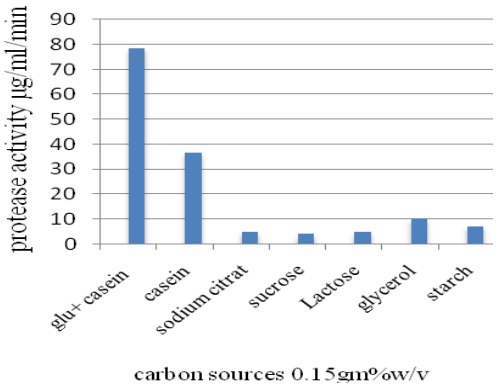


Fig.13 Effect of Nitrogen Sources on Protease Production By A1

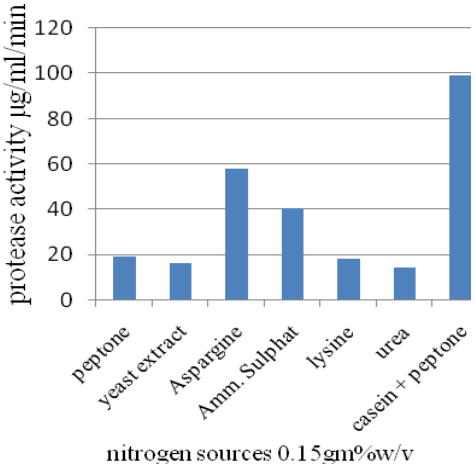


Fig.14 Enhanced Protease Productions in Optimized Production Medium

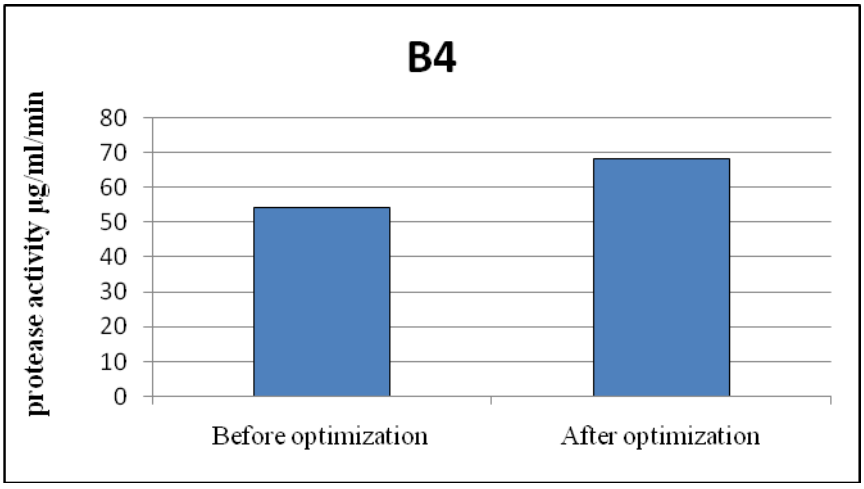


Fig.15 Hydrolysis of Gelatin Coating at Different Time Interval: X-Ray Film After, 1.5h Incubation with Protease At 37°C By B4.

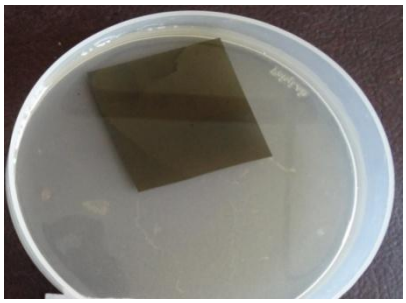
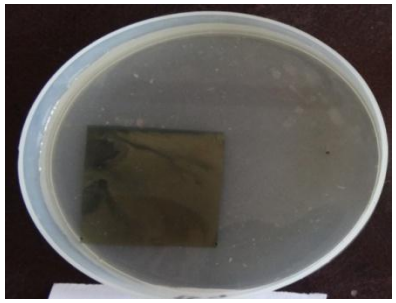
<table>
<tr><td>  </td><td>  </td><td rowspan="2">  </td></tr>
<tr><td>A: Control without enzyme</td><td>B: X-ray film after 60 min</td></tr>
<tr><td>  </td><td>  </td><td rowspan="2"></td></tr>
<tr><td>C: X-ray film after, 90 min</td><td>D: X-ray film after, 180 min</td><td>Transparent and clean X-ray film</td></tr>
</table>

Fig.16 Increase in Protein Content of Hydrolysate

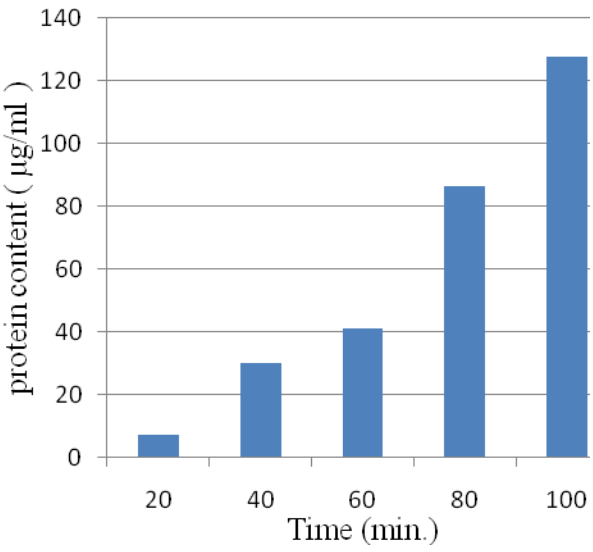
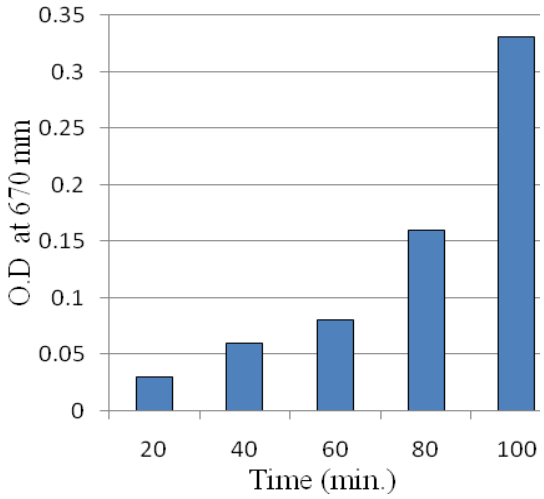


Fig.17 Increase in Hydrolysate Turbidity Suggesting Silver Recovery



The results showed (Figure-9) that the enzyme production was maximum at pH 9.5.(64.54units/ml) Our result matches with the reports for *Bacillus licheniformis* by M.Roja Rani *et al.,* 2012 where growth and protease production were maximum at pH 9.5

Determining optimum pH for caseinolytic activity of crude protease of B4

The optimum pH for enzyme activity was determined by assaying the activity of crude enzyme at various pH values from 6 to 12 with the use of various buffers and the results revealed that the maximum activity was obtained at pH 10.5 with glycine NaOH buffer.

One of the most significant features of the present crude enzyme was its alkaline nature as it was having pH optima of 9.0 so the protease produced by B4 is belonging to alkaline protease group which was confirmed by this exercise. This results were similar to that deduced from several Streptomyces strains (Hames-Kocabas, 2007) and Streptomyces albidoflavus alkaline protease enzyme exhibited optimum activity at pH 9 (Hanaa *et al.,* 2010). The protease of B. licheniformis NH1 has also been reported to have similar properties (Hmidet *et al.,* 2007. The optimum pH range of alkaline protease is generally pH 9 to 11 (Maal *et al.,* 2009; Singh *et al.,* 2001 and Joshi, 2007), with few exception pH 11.5 (Takami *et al.,* 1989; Gessesse and Gashe, 1997) pH 11-12 (Takami *et al.,* 1990), pH 12-13 (Fujiwara *et al.,* 1993).

Effect of Inoculum Size on Protease Production by Isolate B4

Inoculum size also affects the enzyme production greatly (Hameed *et al.,* 1999). Different inoculum sizes represented graphically (Figure: 11) were investigated for their effect on productivity of the protease by B4.

The results indicated that the use of 3.0 ml of 48 h old inoculum (optical density 0.75 at 660 nm), gave the highest yield. Similar result was also found for Streptomyces pulvereceus MTCC 8374 by D.Jayasree *et al.,* 2009, It is well documented that an inoculum size of 2% to 5% is optimum for protease production (Kanekar *et al.,*

2002).Moreover, in the reports of Sinha and Satyanarayana (1991) and according to Gajju *et al.,* (1996) range of 1% to 8% inoculums was the optimum.

Effect of Carbon Sources on Protease Production by isolate B4

There are several reports that different carbon sources have different influences on extracellular enzyme production by different strains (Chi and Zhao, 2003; Beg *et al.,* 2003; Kanekar *et al.,* 2002). Increased yields of alkaline proteases were reported by several workers who used different Sugars such as lactose for Aspergillus flavus (Malathi *et al.,* 1991), maltose for Thermoactinomyces sp. HS682. (Tsuchiya *et al.,* 1991), sucrose for Conidiobolus coronatus NCL 86.8.20 (Phadatare *et al.,* 1993) and fructose for *Bacillus licheniformis* (Sen *et al.,* 1993). However, a repression in enzyme synthesis was observed with these ingredients at high concentrations. Similarly, maximum alkaline protease secretion was observed in *Thermomonospora fusca* YX, which used pure cellulose (Solka-floc) as the principal carbon source (Gusek *et al.,* 1988). Therefore, the effect of different carbon sources on alkaline protease production by B4 was investigated and presented in Figure: 12. The result showed that the best carbon source was mixture of glucose and casein with an activity of 78.55 µg/ml/min. followed by only casein with 36.72 µg/ml/min.

Effect of nitrogen sources on protease production by isolate B4

Alkaline protease production depends heavily on the availability of nitrogen sources in the medium, which has regulatory effects on enzyme synthesis (Patel *et al.,* 2005). Effect of various nitrogen sources for maximum protease production by the isolate was determined and presented in Figure 3.13. Production was optimum with mixture of casein and peptone (98.96 units/ml), followed by aspargine, ammonium sulphate and casein (58.15, 40.81 and 36.72 units/ml respectively) when single nitrogen source was used. Similar reports were also found for Bacillus aryabhattai K3 by K.M. Sharma, *et al.,* 2014.

Protease production in optimized medium and environmental parameters

Result of All above work can be summarized in Table-6.

By considering all optimized conditions and medium components, the final optimized medium was decided and used in further study for the production of protease by B4. The optimized

The optimized medium components and conditions gave 1.26 fold increase in protease production with compared to initially used ordinary production medium, as shown in Figure: 14.

Applications

Enzymatic hydrolysis of gelatin and bioprocessing of used x-ray films for silver recovery.

To determine the efficiency to hydrolyze the gelatinous coating on X-ray film, 68.37U /ml of crude protease was incubated at 37 °C with used X-ray films. Hydrolysis was complete within 2 h (Figure: 15).It was observed that enzyme started hydrolyzing gelatinous coating within 1.5h incubation at 37°C. Finally after 2h It was found that 100% gelatin coating was hydrolyzed and the film was transparent and clean.

Masui *et al.*, (1999) reported 60 min for the complete hydrolysis of gelatin layer; our enzyme completely hydrolyzed gelatin within 90 minutes. Concentration of protein was directly proportional to the rate of gelatin hydrolysis (in experimental hydrolysate) and initially the total protein found was 7.5 µg/ ml. and after 100 minutes it was 127.5 µg/ ml. Figure: 3.16.

The enzymatic hydrolysis of gelatin layers of X-ray films favors the release of silver particles. The alkaline proteases of Bacillus sp. B18′ (Fujiwara *et al.*, 1991) and B. coagulans PB-77 (Gajju *et al.*, 1996) were also efficient in decomposing the gelatinous coating on used X-ray films from which the silver could be recovered. The waste X-ray/ photographic films contain 1.5 - 2 % (w/w) black metallic silver which is recovered and reused. Around 18-20% of the world's silver needs are supplied by recycling photographic waste. Since silver is linked to gelatin in the emulsion layer, it is possible to break the same and release the silver using proteolytic enzymes. At the end of the treatment, gelatin layer was completely removed leaving the polyester film clean and silver was recovered in the hydrolysate, both of which can be reused. (Shankar *et al.*, 2010).Increase in blackening of buffer in the photographs clearly indicated the progress of gelatin hydrolysis along with silver salts, (Figure: 16).The same was analyzed by measuring optical density (at 660 nm) of the hydrolysate at different time interval (Figure: 17).

Acknowledgements

We are thankful to UGC, India for provide financial support to carry out the project work in the form of minor research project. We are also thankful to Gujarat state Biotechnology Mission, Gandhinagar for 16S r RNA analysis of isolates and helping in confirm identification of isolate.

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How to cite this article:

Shilpa A. Jani, Yesha M. Parekh, Tanvi N. Parmar, Tulsi J. Dalwadi, Hetal B. Patel and Sagar K. Parmar. 2016. Screening and Characterization of Alkaline Protease Producing Bacillus Strain B-4 *Bacillus flexus* and Study of its Potential for Alkaline Protease Production. *Int.J.Curr.Microbiol.App.Sci.* 5(5): 767-787.
doi: http://dx.doi.org/10.20546/ijcmas.2016.505.078