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Research Article

USING BACILLUS SP FOR THE PRODUCTION OF ALKALINE PROTEASE BY SUBMERGED FERMENTATION

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ABSTRACT

Alkaline protease is the most important group of protease enzymes utilized commercially in various arenas of industries, such as food, detergent, leather, dairy, pharmaceutical, diagnostics, and waste management. Microorganisms of specially those belonging to *Bacillus* genera serve as a vast repository of diverse set industrially important enzymes and utilized for the large scale enzyme production using fermentation technology.

Bacteria were isolated from soil samples from Parangipettai in India using serial dilution method. Different isolates were then screened for their alkaline protease producing capability using skim milk hydrolysis test. Isolates showing positives result for the skim milk hydrolysis test were identified based on their morphology by staining and their biochemical properties. Based on bio-informatic analysis thus identified five different Bacillus species (*Bacillus megaterium*, *Bacillus pumilus*, *Bacillus subtilis, Bacillus chonii* and *Bacillus cereus)* were identified.

Eight fermentation media were used for production of Alkaline protease for identifying the media in which yield is maximum for the Alkaline protease enzyme production media (MD8) produced highest amounts of alkaline protease (62.5 U/mL).

The study also aimed at determining the most optimized conditions for Alkaline protease production and activity from five different species of *Bacillus* like pH, metal ions, nitrogen source, carbon source and NaCl. The most optimized media and culture conditions will help to enhance the protease production which could play major role in various detergent industries.

Keywords: Enzyme, Protease, Bacillus Sp. Fermentation, Purification

INTRODUCTION

Microorganisms are known to play a vital role in technology for the production of intracellular and extracellular enzymes on an industrial scale (Gupta *et al*., 2007, Moo-young & Chisti, 1994). For maximum yield, selected organisms are grown in fermenters under optimum conditions and can be further used to make products such as cheese, bread, wine and beer (Lin *et al.,* 2006, Brijwani *et al.,* 2010, Schenk *et al.,* 2008). Most reactions inside living cells require enzymes, which act as catalysts and are essential for life (Pingoud et al., 2005).

Bacillus species are the main producers of extracellular proteases, and industrial sectors frequently use *Bacillus subtilis* for the production of various enzymes. Proteases are the main enzymes produced from microbial sources, of which only few are recommended as commercial producers. *B. subtilis* is found mainly in soil and is also known as hay bacillus and grass bacillus. It is a rod-shaped organism, which can form a tough, protective endospore and can with stand extreme environmental conditions. *Bacillus* species are obligate aerobes or facultative anaerobe and include both free-living and pathogenic species. In view of their wide application in various industries, protease enzymes occupy an important position (Widsten, and Laccase, 2008).

Alkaline proteases constitute 60–65% of the global industrial market (Sawi *et al.,* 2008). These proteases are the single class of enzymes widely used in detergents, pharmaceuticals, leather and the food and agriculture industries (Azura *et al.,* 2009). *B. subtilis* is widely used for the production of specific chemicals and industrial enzymes. Proteases are important components of biopharmaceutical products such as contact lens cleaners and enzymatic debriders. Proteolytic enzymes support the natural healing process in local management of skin ulceration by efficient removal of necrotic material (Sjodahl *et al*., 2002).

Proteases catalyze or hydrolyze protein and therefore play a vital role in various industrial applications. Hyperactive strains are being sought for use in different industries Conversion of wastes into useful biomass by microorganisms and their enzymes is a new trend, and new protease-producing microorganisms and perfected fermentation technology are needed to meet the ever-growing demand for this enzyme (Rathakrishnan *et al.,* 2012). The purpose of this work was to study to production of alkaline protease enzyme from five different strains of *Bacillus bacteria* by using submerged fermentation.

MATERIALS AND METHODS

Isolation of bacteria:

Soil sample was collected from Parangipettai, Cuddalore in India were used for isolation of *Bacillus* species by serial dilution method. 1 g of the sample was suspended in 100 ml sterile distilled water, agitated for 45 min on a shaker at 50°C and 0.2 ml was spread on casein agar plates (nutrient agar with 1% casein) and incubated at 30°C for 7 days. Enriched sample was plated over nutrient agar containing 0.4% gelatin (Harrigan *et al*., 1966). After incubation for 24 h, plates were flooded with 1% tannic acid. Colonies showing clear zone

were picked and purified. A total of 75 isolates were screened for protease production by using casein digestion method. Five isolates BS1,BS2,BS3,BS4 and BS5 which showed maximum activity were selected and maintained on nutrient agar at 4°C.(composition of nutrient agar and broth media, serial dilution, spreaded plates and streaked plates shown in Appendices 1,2,3,4 and 5 respectively).

Identification of the microorganism using different tests:

The isolates were identified by Gram staining, and biochemical tests like Sugar fermentation, citrate utilization, catalase, methyl red/voges-prokaure, urease test, starch hydrolysis, and indole tests.

Estimation of Alkaline protease by DNS Assay:

The steps of estimation of alkaline protease by DNS (Dintro Salicylic Acid) Assay. Pipetted in the following reagents into a series of dry clean and labelled test tube and as indicated, as shown in section A. After replaced the above mentioned solution as in section A in the labelled tubes, shake well and then placed them in a boiling water bath for 5 minutes. And there after cooled thoroughly at room temperature and then added 7.0ml of distilled water to each tube as indicated in section B, read the extinction (optical density) of the coloured solution at 540nm used the solution in tube 1 as a blank (control). Since extenction is sensitive to temperature change. Tubes were cooled at room temperature only. Recorded the reading in section B, and plotted the relationship between the optical density and the concentration of glucose solution. To evaluate whether there is a liner relationship between the concentrations of glucose solutions and their corresponding optical density. Prepared standard curves of the sugars provided and used them to estimate the concentration of alkaline protease enzyme.

Fermentation media:

Different production media were used for the production of alkaline protease. The pH of each of the following media was adjusted to 9 with 1N HCl and 1N NaOH solution and the media containing 100ml each was sterilized

Alkaline Protease Production:

Eight different enzyme production media were used for protease production. Culture suspension was inoculated in 250 ml of Erlenmeyer flask containing 100 ml culture medium. The medium was incubated for 48 h in shaker incubator at 37 °C. After 48 hours, the fermented broth was centrifuged at 10,000 rpm for 10 minutes to extract the crude extracellular Protease. The pellet which contains majorly the cell mass and debris was discarded and supernatant was collected. The supernatant was further used as crude enzyme extracts, used for enzyme assay and quantification.

Proteolytic Activity Assay:

The assay of alkaline protease was carried out taking crude enzyme in series of tubes. Substrate was then added to each tube and kept in dark for 15 minutes. Then DNS reagent (Dintro Salicylic Acid) was added, incubated in water bath. Absorbance was then read at 540 nm.

Screening for Alkaline protease activity:

The alkaline protease activity was estimated by the procedure of modified Hagihara method using casein as substrate (Hagihara *et al.,* 1958). For this, the pure cultures obtained and identified were streaked on Alkaline Skim milk agar plate (Skim milk 1.0%, Peptone 0.1%, NaCl 0.5%, Agar 2.0% and pH 10.0). The Alkaline Protease production of the selected bacterial colony was confirmed by the formation of clear zones around the colonies. The isolate with maximum zone formation was maintained onto nutrient agar slant.

Protein concentration estimation:

The protein concentration was determined by the Lowry method **(Lowry *et al.,* 1951)** using sodium carbonate, sodium hydroxide and Folin's Ciocalteau reagent. The colour change was measured at 600 nm using colorimeter. The protein concentration was estimated by comparing the value with standard graph prepared using Bovine serum albumin as standard

Purification of alkaline protease:

Ammonium sulphate fractionation:

The Alkaline protease enzymes were also purified by slaying. The protein fraction precipitated with 85 % ammonium sulfate. The suspension was centrifuged at 10,000 rpm for 15 min ,the precipitate was collected and dissolved in 25 M NaCl; 10M Tris base buffer Ammonium sulfate was found to activate the protease activity after dialysis.

Dialysis: The precipitate obtained after ammonium sulphate precipitation was dissolved in 25mM NaCl; 10M Tris base buffer. Then it was put inside a semi permeable membrane bag and kept immersed in Tris base buffer for dialysis at 4°C. After dialysis the enzyme was taken out 31 and centrifuged at 8000 rpm for 15 minutes. The supernatant is collected as the pure enzyme after dialysis.

Column chromatography:

The precipitated enzyme was then subjected to Column chromatography with Silica Gel as the stationary phase, 0.1M phosphate buffer (pH 7.0) as Running buffer and 1M NaCl as elution buffer (pH 7.0) for further purification.

Alkaline protease estimation:

For estimation of Alkaline protease casein solution was used as a substrate. Casein solution and buffer solution (carbonate-bicarbonate buffer) were taken in test tube and further enzyme solution (cell extract) was also added in test tube and the reaction mixture thus prepared, was incubated. The reaction was stopped by adding trichloroacetic acid solution. After filtration of this solution, filtrate was used for further experiment. Then after, small amount of aliquot and Na_2CO_3 was taken and C-reagent was added in the test tube. After few minutes follin reagent (double diluted) was added in the test tube. After 30 minutes the O.D. was estimated.

RESULTS

Isolation of bacteria:

Isolation of bacteria was done from soil sample by serial dilution method (Benson, 2002).10^{-6} dilution of soil sample was spread onto Nutrient agar media and incubated for 24 hours at 37ºC and pure culture was obtained by spreading the sample on nutrient agar media and streaking for obtaining the pure culture.

Isolation of bacterial pure culture:

Pure culture was then obtained on Nutrient agar media by streaking the desired colonies.

Identification of Isolated Bacterial Species:

Identification of the isolated bacterial species were done by performing biochemical tests (Sugar Fermentation, citrate utilization, catalase, methyl red/ voges prokaure, urease test, starch hydrolysis, and indole tests) as shown in the Table 1.

S. No	Biochemical test	Strains				
		BS1	BS2	BS3	BS4	BS5
1	Gram staining	Gram +ve	Gram +ve	Gram +ve	Gram +ve	Gram +ve
2	Morphology	Rod	Rod	Rod	Rod	Rod
3	Sugar fermentation Glucose Sucrose Mannitol	 +ve +ve -ve	 +ve -ve -ve	 -ve -ve +ve	 +ve +ve +ve	 -ve -ve +ve
4	Citrate utilization	+ve	+ve	+ve	+ve	+ve
5	Casein hydrolysis	+ve	+ve	+ve	+ve	+ve
6	Catalase test	+ve	+ve	+ve	+ve	+ve
7	MR/VP test	+ve/-ve	+ve/-ve	+ve/-ve	+ve/-ve	+ve/-ve
8	Urease test	+ve	+ve	+ve	+ve	+ve
9	Starch hydrolysis test	+ve	+ve	+ve	+ve	+ve
10	Indole test	-ve	-ve	-ve	+ve	-ve

Table 1: Biochemical results for isolates

Estimation of Alkaline Protease Yield by BS1 Sample:

In BS1 sample (*Bacillus cohnii*) the maximum enzyme activity was shown by media MD8 (62.5U/ml) followed by media MD7 (46.87U/ml), MD4 (42.96U/ml), MD5 (38.28U/ml), MD2 (25U/ml), MD1 (21.875U/ml) respectively and minimum enzyme activity was shown by media MD6 (14.06U/ml). In BS2 sample (*Bacillus subtilus*) the maximum enzyme activity was shown by media MD8 (59U/ml) followed by media MD7 (46.87U/ml), MD4 (46.26U/ml), MD5 (37.95U/ml), MD3 (28.9U/ml), MD1 (18.75U/ml) respectively and

minimum enzyme activity was shown by media MD6 (9.23U/ml). In BS3 *(Bacillus cerus*) the maximum enzyme activity was shown by media MD7 (44U/ml) followed by media MD8 (42.96U/ml), MD4 (32U/ml), MD5 (29.64U/ml), MD3 (25.42U/ml), MD2, (22.32U/ml), MD6 (14.46U/ml) respectively and minimum enzyme activity was shown by media MD1. In BS4 sample (*Bacillus pumilus*) the maximum enzyme activity was shown by media MD8 (63.21U/ml) followed by media MD7 (39.67U/ml), MD5 (33.33U/ml), MD4 (24.16U/ml), MD2 (23.23U/ml), MD3 (18.9U/ml), MD6 (11U/ml) respectively and minimum enzyme activity was shown by media MD1. In BS5 sample *(Bacillus megaterium*) the maximum enzyme activity was shown by media MD8 (46.71U/ml) followed by media MD4 (36.09U/ml), MD7 (32.11U/ml), MD5 (28.83U/ml), MD1 (13.01U/ml), MD2 (12.94U/ml), MD3 (11.11U/ml) respectively and minimum enzyme activity was shown by media MD6 (11.01U/ml).

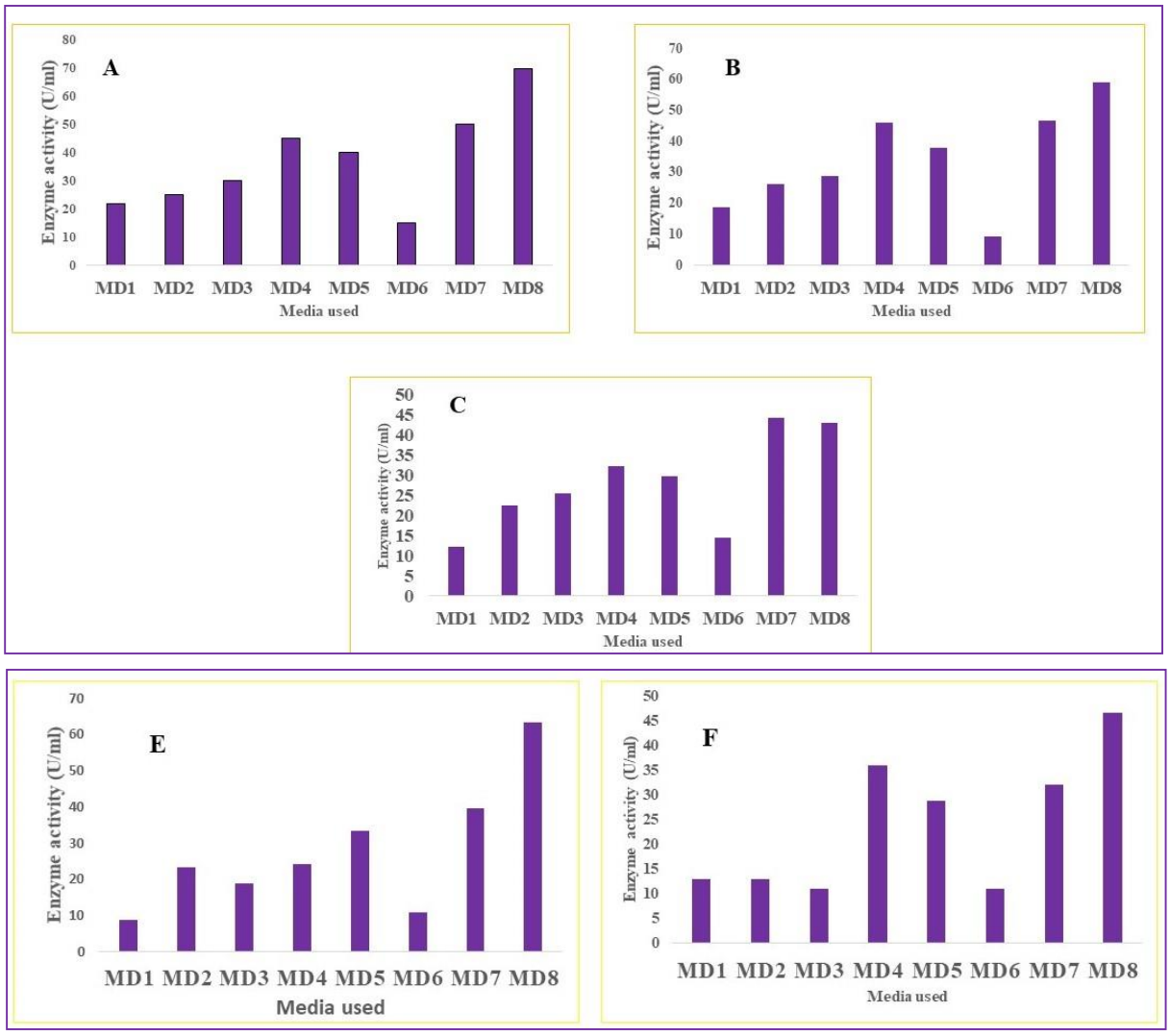

Figure 1: Yield of Alkaline Protease for BS1 to BS5 in 8 different media (A-BS1, B-BS2, C-BS3. D-BS4 and E-BS5.

Quantification of Enzyme using Lowry's Assay:

A standard curve was plotted for known concentrations of reference protein (Bovine Serum Albumin). Figure 2. Concentrations of the purified enzyme were then calculated using the standard curve of BSA.

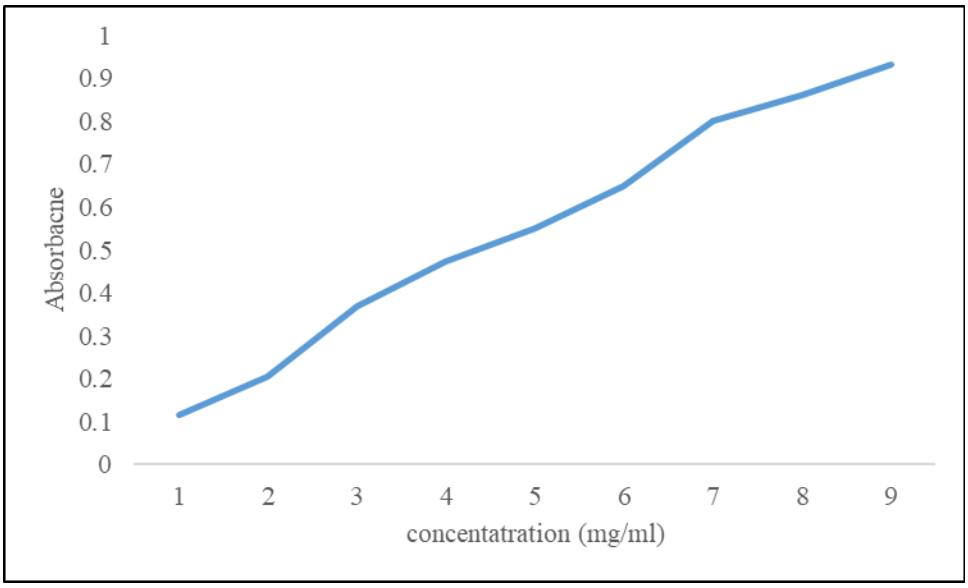

Figure 2: A standard curve of Lowry's Assay for BSA

Purification of Alkaline Protease Enzyme:

Purification of Alkaline Protease using Column Chromatography:

The precipitated enzyme was then purified on a stationary bed of Silica gel and eluted out using phosphate buffer. The collected fractions were then quantified using Lowry's method.

Purification of Alkaline Protease using Ammonium Sulphate Precipitation:

Three percentages of ammonium sulphate solution was used to separate the enzyme from the solution. The percent of solution used were 30%, 50% and 70%. Maximum precipitation of enzyme was observed with 50% salt treatment for BS1,BS3 and BS4 while the concentration of salt was observed in the case of 70% in case BS2and BS5.

Quantification of Enzyme:

The maximum concentration of enzyme by 30% ammonium sulphate precipitation was shown to BS1 sample followed BS5 sample and minimum concentration was showing to BS2, BS3, BS4 samples at same amount of concentration, as shown in Table 2 and Figure3.

The maximum concentration of enzyme by 50% ammonium sulphate precipitation was shown to BS1sample followed by BS4 sample and minimum concentration was showing to BS5sample, as shown in Table 2 and Figure 3.

The maximum concentration of enzyme by 70% ammonium sulphate precipitation was shown to BS1samlefollowed by BS2 sample and minimum concentration was showing to BS4samle, as shown in Table 2 and Figure 3.

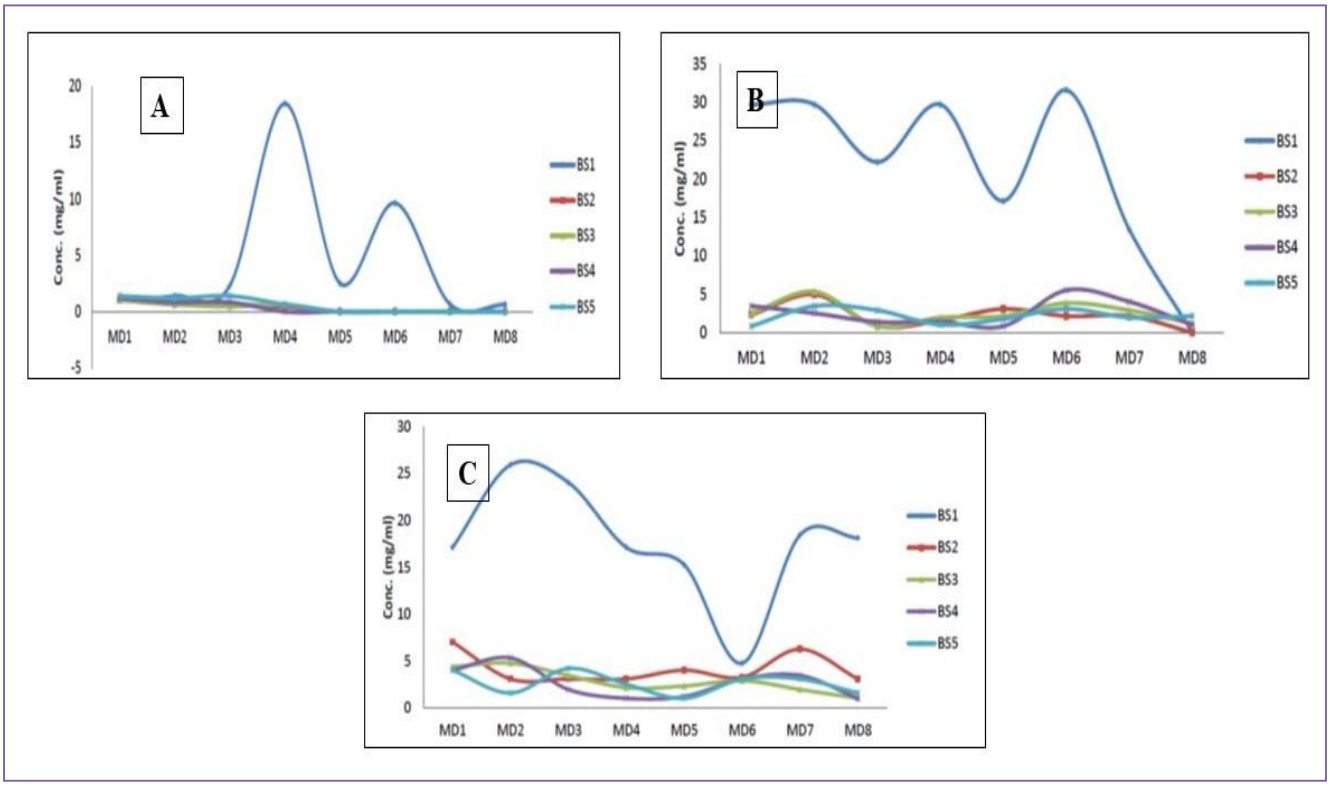

Figure 3: Concentration of enzyme at ammonium sulphate precipitation (A-30%, B-50 %, C-75%)

Media	Samples									
	BS1		BS2		BS3		BS4		BS5	
	30% Ammonium sulphate precipitation									
	OD	Con	OD	Con	OD	Con	OD	Con	OD	Con
MD1	0.07	1.01	0.07	1.02	0.07	1.2	0.08	1.2	0.09	1.39
MD2	0.09	1.39	0.05	0.64	0.05	0.64	0.06	0.83	0.08	1.2
MD3	0.14	2.32	0.05	0.45	0.04	0	0	0.83	0.09	0.64
MD4	1	18.44	0.04	0	0.05	0	0	0	0.05	0
MD5	0.05	2.51	0	0	0	0	0	0	0	0
MD6	0.53	9.63	0	0	0	0	0	0	0	0
MD7	0.05	0.64	0	0	0	0	0	0	0	0
MD8	0.05	0.64	0	0	0	0	0	0	0	0
	50% Ammonium sulphate precipitation									
MD1	0.37	6.64	0.14	2.33	1.5	2.51	0.2	3.45	0.06	0.83
MD2	1.6	29.68	0.28	4.95	0.36	5.33	0.15	2.51	0.2	3.45
MD3	1.6	29.68	0.06	4.95	0.06	0.83	0.09	1.39	0.17	2.89
MD4	1.2	22.19	0.1	0.83	0.12	1.95	0.09	1.39	0.07	1.01
MD5	1.6	29.68	0.18	1.58	0.13	2.4	0.06	0.83	0.11	1.16
MD6	0.93	17.13	0.13	3.07	0.22	3.83	0.31	5.51	0.18	3.07
MD7	1.7	3.56	0.13	2.14	0.17	2.89	0.23	4.01	0.12	1.95
MD8	0.13	13.38	0	0	0.08	1.2	0.07	0.13	0.13	2.14
	75% Ammonium sulphate precipitation									
MD1	0.93	17.13	0.39	7.01	0.28	4.39	0.23	4.01	0.23	4.01
MD2	1.4	25.94	0.18	3.08	0.27	4.16	0.3	5.32	0.1	1.58
MD3	1.3	24.06	0.18	3.08	0.2	3.45	0.12	1.95	0.24	4.2
MD4	0.93	17.13	0.18	3.08	0.13	2.14	0.07	1.01	0.15	2.51
MD5	0.83	15.26	0.23	4.4	0.14	2.32	0.08	1.2	0.07	1.01
MD6	0.27	4.16	0.19	3.26	0.17	2.89	0.18	3.07	0.18	3.07
MD7	1	18.44	0.35	6.26	0.12	1.95	0.2	3.45	0.18	3.07
MD8	0.45	18.13	0.18	3.07	0.07	1.01	0.07	1.01	0.1	1.58

Table 3: Depicting the concentration of precipitated enzyme using Lowry's Assay

DISCUSSION

Alkaline proteases possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures. In leather industries alkaline proteases are exhibiting a prominent role in unhairing and bating processes (Sjodahl, *et al.,* 2002).

Alkaline protease was produced by different spcies of *Bacillus* using submerged fermentation (Karadag *et al.,* 2009). Five different species of *Bacillus* were isolated from the soil from Lucknow in India, cultured and screened for their alkaline protease production from different strains like BS1, BS2, BS3, BS4 and BS5 (Kumar and Vats 2010).

The bacterial species1 (BS1) was thus identified as *Bacillus cohnii* with highest score of 2579 and % identity of 99% with the query sequence. The bacterial species 2 (BS2) was thus identified as *Bacillus subtilis* with highest score of 2429 and % identity of 97% with the query sequence (Vijayaraghavan, *et al.,* 2014). The bacterial species 3 (BS3) was thus identified as *Bacillus cereus* with highest score of 1934and % identity of 96% with the query sequence and the bacterial species 4 (BS4) was thus identified as *Bacillus pumilus* with highest score of 2217 and % identity of 94% with the query sequence. The bacterial species 5 (BS5) was thus identified as *Bacillus megaterium* with highest score of 2333 and % identity of 96% with the query sequence (Starcher, 2001).

It was observed that MD8 was found to be the most suitable medium for alkaline protease production from all samples in general i.e: BS1 (62.5 U/mL), BS2 (59U/ml), BS3 (42.96U/ml) BS4 (63.21U/ml) and BS5 (46.71U/ml).and MD6 turned out to be minimum productive for alkaline production as its enzyme activity was found minimum (14.06 U/mL) (Rao, et al., 2008).

The extracted crude enzyme was then precipitated using varied percentages of ammonium sulphate (between 30% to 75%) (Fu *et al.,* 2007). The precipitated sample was dissolved in phosphate buffer and loaded to Silica gel column. Quantification was done for total protein content by Lowry's method. It was found that maximum precipitation to BS1 was observed with 50% ammonium sulphate solution in the case MD7(31.56mg/ml),BS2 was observed with 70% ammonium sulphate solution in the case MD1(7.01mg/ml), BS3 was observed with 50% ammonium sulphate solution in the case MD2(5.33mg/ml), BS4 was observed with 50% ammonium sulphate solution in the case MD6 (5.51mg/ml) and BS5 was observed with 70% ammonium sulphate solution in the case MD3 (4.20mg/ml) (Schallmey *et al.,* 2004). Thus highest concentration of protein was obtained in the case of MD2 (29.68 mg/ml) MD3 (29.68 mg/ml), MD4 (22.19 mg/ml), MD5 (29.68 mg/ml), MD6 (17.13 mg/ml), MD7 (31.56 mg/ml) and MD8 (13.38 mg/ml) while lesser in case of MD1 (6.64 mg/ml) where more precipitation was observed with 70% ammonium sulphate solution. The impact of media components and its environmental conditions play a major role in the final yield of Enzyme at the end of fermentation process (Anwar, 2000).

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