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RESEARCH PAPER

Isolation, identification, partial purification, optimization and characterization of alkaline protease from *Neisseria flavescence*

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Microbial alkaline proteases are among the important hydrolytic enzyme and have been used extensively since the advent of enzymology. Bacterial extracellular alkaline proteases are of great importance due to its wide spectrum applications in detergent industries, bioremediation, food industries, and leather processing and bio-film degradation. From the various niches eighteen isolates were screened for alkaline protease production, out of which four isolates showed efficient alkaline protease production. Out of four bacterial species one of the isolate *i.e. N. flavescence* showed significant enzyme activity. Optimization of the pH and temperature conditions for enzyme production was determined and was found to be 7.0 and 60^{0}C, respectively. Optimization of carbon, nitrogen sources and metal ions for enzyme production were determined and was found to be 4.64U/ml for sucrose, 0.91U/ml for gelatin and 0.21U/ml for zinc chloride for the isolates. The yield of alkaline protease was inhibited by copper sulphate. Enzyme activity was assayed using tyrosine-casein method. The purified enzyme preparations of having enzyme activity 0.26U/ml was also excellent in destaining of ink colour. The molecular weight of different bands of alkaline protease from *N. flavescence* ranged from 25kD to 83kD.

Key words : *Neisseria flavescence*, Alkaline protease, SDS, Sucrose, Gelatin, Zinc chloride, Copper sulphate

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INTRODUCTION

With increasing emphasis on environmental protection, the use of microbial enzymes particularly from extreme-philes has gained considerable attention during the last several years in many industries, including manufacturing of chemicals, textiles; pharmaceuticals, paper, food and agriculture chemicals (Mehta *et al.,* 2006).Alkaliphiles are reported to be a rich source of alkaline active enzymes, for example, amylase, protease, cellulase, xylanase and other enzymes that have numerous applications in many industrial processes due to an interest in their physiological adaptation to high pH (Oskouise *et al.,* 2008).

Proteases are one of the most important industrial en-zymes accounting for nearly 60% of the total world-wide enzyme sales (Ward, 1985; Kalisz, 1988; Beg *et al.,* 2003; Ellaiah *et al.,* 2003; Adinarayana and Ellaiah, 2003; Moreira *et al.,* 2003).Proteolytic enzymes are degradative enzymes which catalyze the cleavage of peptide bonds in other proteins. Alkaline proteases, which are referring to proteolytic enzymes which work optimally in alkaline pH are the main enzymes among proteases and constitute 60 to 65 per cent of the global industrial

enzyme market (Amoozegara *et al.,* 2007). Moreover, they are used in the food industry in meat tenderization processes, peptide synthesis, infant formula preparations, baking and brewing. They are also used in the detergent industry as additives, in pharmaceutical and medical diagnosis as well as in the textile industry in the process of dehairing and leather processing (Tari *et al.,* 2006; Bhaskar *et al.,* 2007; Dodia *et al.,* 2008).Alkaline proteases are of great interest because of their high proteolytic activity and stability under alkaline conditions (Maurer, 2004; Saeki *et al.,* 2007).These enzymes find applications in detergents, feather processes, food processing, silk gumming, pharmaceuticals, bioremediation, biosynthesis and biotransformation (Bhaskar *et al.,* 2007; Gupta *et al.,* 2002; Jellouli *et al.,* 2009; Sareen and Mishra, 2008). The majority of commercial alkaline proteases are produced by bacteria (Jellouli *et al.*, 2009). Since the first alkaline protease Carlsberg from *Bacillus licheniformis* was commercialized as an additive in detergents in the 1960s (Saeki *et al.,* 2007), a number of Bacillus-derived alkaline proteases have been purified and characterized and significant proteolytic activity, stability, broad substrate specificity, short period of fermentation, simple downstream purification and low cost have been demonstrated (Haddar *et al.,* 2009; Maurer, 2004).The main objective of this project was to study the effect of nutritional and environmental parameters of alkaline protease production and determination of its molecular weight.

RESEARCH METHODOLOGY

Isolation and screening of bacteria for protease production:

Bacterial species was isolated from soil sample using serial dilution methods. Identification of selected isolate was studied based on different morphological and biochemical characteristics. The data were compared with standard description given in Bergy's Manual of Systematic Bacteriology. The isolated bacterial species was screened for the protease production *viz.,* skim milk agar media.

Production and estimation of alkaline protease :

Production of alkaline protease was done in eight different fermentation media inoculated with isolated bacterial species for three days. After alkaline protease production the estimation of enzyme was done using casein as substrate and carbonate-bicarbonate buffer (sodium carbonate - sodium bicarbonate buffer solution).

Partial purification and SDS-PAGE :

The isolated enzyme was partially purified by gel filtration chromatography *viz.,* sephadex G-75 and the molecular weight was determined through SDS-PAGE technique.

Optimization of alkaline protease production :

The production of alkaline protease enzyme in all the eight different fermentation media was optimized at different parameters like pH (3, 5, 7, 9 and 11), temperature (40°C, 50°C, 60°C, 70°C and 80°C), carbon source (lactose, sucrose, fructose, maltose and dextrose), nitrogen source (gelatin, ammonium chloride, urea, ammonium nitrate and sodium nitrate) and metal ions (sodium chloride, potassium chloride, calcium chloride, magnesium chloride and zinc chloride).

Characterization of alkaline protease :

The characterization of enzyme was done by the study effect of inhibitors on alkaline protease production. To study the effect of inhibitors on protease production yield, different inhibitors used were SDS, EDTA, β-mercaptoethanol, ferric nitrate and copper sulphate.

Effect of different concentration of substrate protease production :

In the fermentation media different concentration of casein at the range of 2-10mM was added and incubated with test organism under optimum condition. After incubation at 37°C for 24 hours, the sample were withdrawn and assayed for alkaline protease activity.

RESEARCH FINDINGS AND ANALYSIS

The bacterial species was isolated from soil sample and screened for the production of alkaline protease in skim milk agar media. The result was found to be positive hence, the bacterium was able to produce alkaline protease (Fig. 1).

The estimation of protease activity was done in eight different fermentation media via; casein as substrate and it was found that production media M_6 was best for protease production (Fig. 2). The enzyme was further purified by gel filtration chromatography using sephadex

Fig. 1 : Positive result of casein hydrolysis test (*Neisseria flavescence*); (i) Clear zone around the growth due to production of protease enzyme (ii) Media used : Skim milk agar media

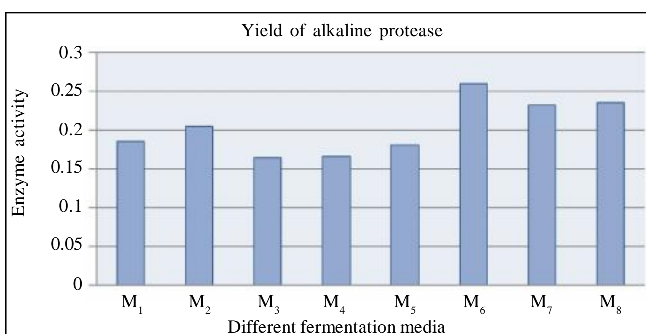

Fig. 2 : Estimation of alkaline protease produced from *Neisseria flavescence* (a) Maximum yield was observed in M_6 fermentation media

G-75 and the fractions were quantified with Lowry's assay to determine the concentration of enzyme. The purification values of extracted, precipitated and purified samples were determined by fold purification (Table 1).

The molecular weight of enzyme isolated from *N. flavescence* was determined by SDS-PAGE using specific markers of known molecular weight. The molecular weight of alkaline protease from *N. flavescence* ranged from 25kDa to 83kDa (Table 2, Fig.3 and 4).

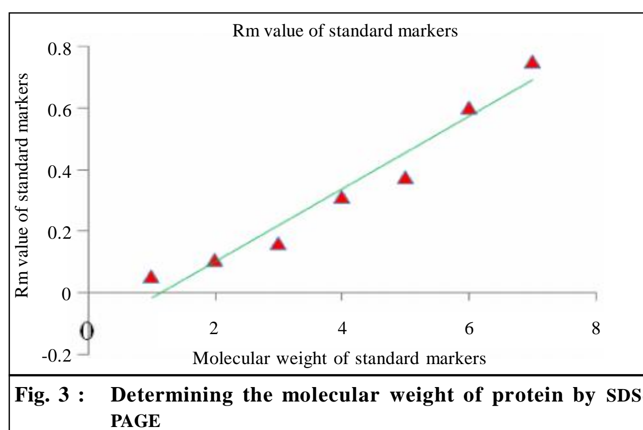

Fig. 3 : Determining the molecular weight of protein by SDS-PAGE

Optimization of pH for alkaline protease production:

All the eight different fermentation media inoculated with *Neisseria flavescence* were optimized at different pH range using sodium carbonate and sodium bicarbonate buffer solution (adjusted at different pH

Table 1 : Fold purification table of alkaline protease via. *Neisseria flavescence*

Volume (cm^3)	Concentration	Total protein	Activity	Total activity	Specific activity	Purification fold	Overall yield
$15cm^3$	8.27	124.05	0.54	8.1	0.065	1	100%
$7cm^3$	7.38	51.66	0.53	3.71	0.071	1.09	45.8%
$4cm^3$	7.74	30.96	0.56	2.24	0.072	1.11	27.6%

Table 2 : Molecular weight from SDS (co-omassie staining) gel of *N. flavescence* alkaline protease

Sr. No.	M.W. of marker	Distance of marker	Distance of protein band	M.W. of protein band
1.	250kd	0.5cm	1.2cm	169kd
2.	175kd	1.0cm	3.8cm	83kd
3.	150kd	1.5cm	4.9cm	54kd
4.	100kd	2.9cm	5.5cm	37kd
5.	75kd	3.5cm	6.3cm	29kd
6.	37kd	5.6cm	7.5cm	25kd
7.	15kd	7.0cm	8.0cm	17kd

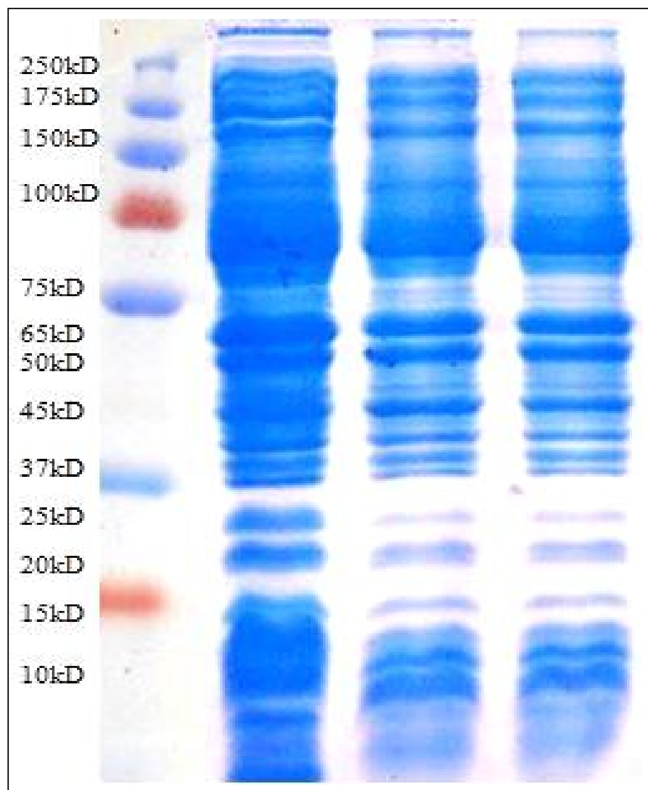

Fig. 4 : Bands of alkaline protease isolated from *N. flavescence* (a) Lane 2-4 shows bands of alkaline protease (b) lane 1 shows ladder from 10kDa to 250kDa

range) to study the maximum activity of alkaline protease. The maximum activity was observed in M_1 (1.5U/ml±0.26), M_4(2.45±0.13U/ml), M_3(3.43±0.41U/ml), M_1 (2.76±0.16U/ml) and M_4 (1.43±0.19U/ml) at pH3, pH5, pH7, pH9 and pH11, respectively. Among these maximum activities was at 7 in M_3 (3.43±0.41U/

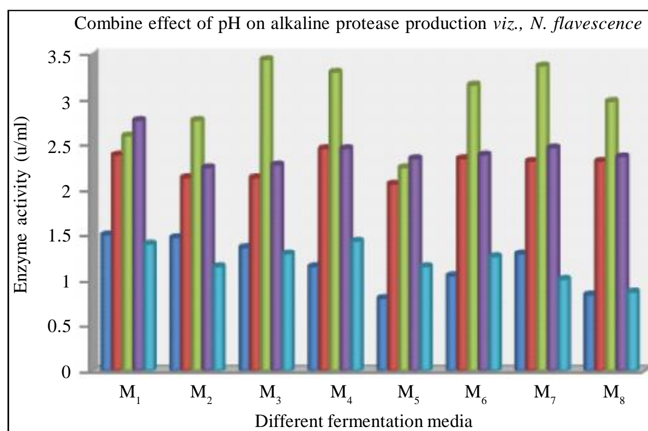

Fig. 5 : Optimization of alkaline protease production from *N. flavescence* at different pH (a) Maximum yield was observed in M_7 at pH 7 (b) Minimum yield in M_5 at pH3. All the values are ANOVA significant at $p<0.05$

ml) media was found to be significant for alkaline protease production (Fig. 5). Most of the commercially available subtilisin-type proteases are also active in the pH range of 8-12 (Gupta *et al.,* 2002).

Optimization of temperature for alkaline protease production from *N. flavescence* :

All the eight different fermentation media inoculated with *N. flavescence* were optimized at different temperature range using sodium carbonate and sodium bicarbonate buffer solution to study the maximum activity of alkaline protease. The maximum activity was observed in M_1 (10.54±0.06U/ml), M_8 (11.00±0.38U/ml), M_1 (11.46±0.50U/ml), M_6 (11.39±0.66U/ml) and M_5 (10.42±0.51U/ml) at 40°C, 50°C, 60°C, 70°C and 80°C, respectively. Among these highest activity was at 60°C in M_1 (11.46±0.50U/ml) media was found to be significant for alkaline protease production (Fig. 6).The optimum temperatures of alkaline proteases ranged from 40 to 80ºC; the alkaline protease was stable for so long even at autoclaving (121ºC) and boiling temperatures (Kaur *et al.,* 2001).

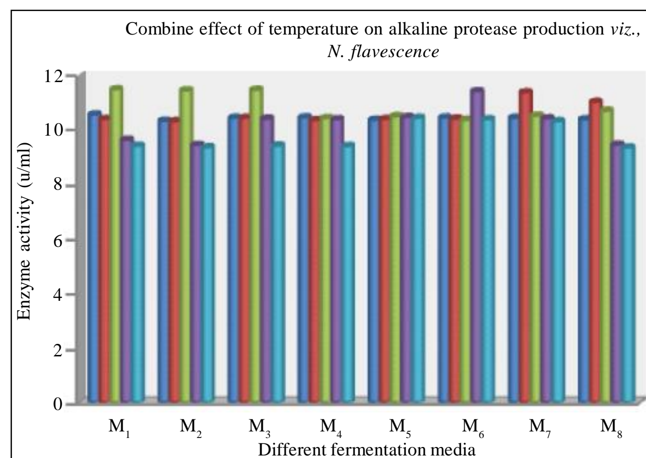

Fig. 6 : Optimization of alkaline protease production from *N. flavescence* at different temperature (40°C, 50°C, 60°C, 70°C and 80°C) (a) Maximum yield was observed in M_1 at 60°C (b) Minimum yield in M_2 at 80°C.All the values are ANOVA significant at $p<0.05$

Optimization of carbon source for alkaline protease production from *N. flavescence* :

All the eight different fermentation media inoculated with *N. flavescence* were optimized by different carbon source using sodium carbonate and sodium bicarbonate buffer solution to study the maximum activity of alkaline protease. The maximum activity was observed in M_1

(4.61±0.56U/ml), M_1 (4.64±0.57U/ml), M_6 (3.79±0.25U/ml), M_1 (4.47±0.61U/ml) and M_1 (4.03±0.52U/ml) in lactose, sucrose, fructose, maltose and dextrose as carbon source, respectively. Among these highest activity was in sucrose in M_1 (4.64±0.57U/ml) media was found to be significant for alkaline protease production (Fig. 7). Among the different carbon sources fructose was found to be best carbon source for production of alkaline protease (Verma *et al.,* 2011).

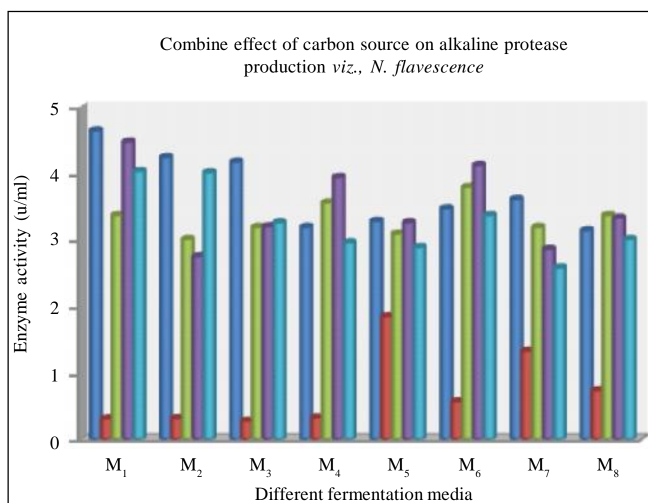

Fig. 7 : Optimization of alkaline protease production from *N. flavescence* in different carbon source (lactose, sucrose, fructose, maltose and dextrose) (a) Maximum yield was observed in M_1 *viz.,* sucrose (b) Minimum yield in M_7 *viz.,* dextrose. All the values are ANOVA significant at $p<0.05$

Optimization of nitrogen source for alkaline protease production from *N. flavescence* :

All the eight different fermentation media inoculated with *N. flavescence* were optimized by different nitrogen source using sodium carbonate and sodium bicarbonate buffer solution to study the maximum activity of alkaline protease. The maximum activity was observed in M_1(0.91±0.31U/ml), M_1(0.13 ± 0.01U/ml), M_8 (0.104 ± 0.03U/ml), M_8 (0.90 ± 0.28U/ml) and M_1 (0.11 ± 0.01U/ml) in gelatin, ammonium chloride, urea, ammonium nitrate and sodium nitrate as nitrogen source, respectively. Among these highest activity was in gelatin in M_1 (0.91 ± 0.31U / ml) media was found to be significant for alkaline protease production (Fig. 8). Among the different nitrogen sources gelatin was found to be best nitrogen source for production of alkaline protease (Verma *et al.,* 2011).

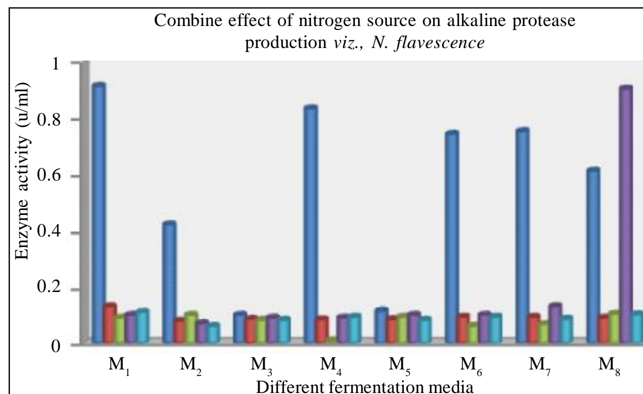

Fig. 8 : Optimization of alkaline protease production from *N. flavescence* in different nitrogen source (gelatin, ammonium chloride, urea, ammonium nitrate and sodium nitrate) (a) Maximum yield was observed in M_1 *viz.,* gelatin (b) Minimum yield in M_4 *viz.,* urea.All the values are ANOVA significant at $p<0.05$.

Optimization of metal ions for alkaline protease production from *N. flavescence* :

All the eight different fermentation media inoculated with *N. flavescence* were optimized by different metal ions using sodium carbonate and sodium bicarbonate buffer solution to study the maximum activity of alkaline protease. The maximum activity was observed in M_1 (0.107±0.01U/ml), M_1 and $_8$ (0.095 ±0.01U/ml), M_8 (0.121±0.01U/ml), M_1 (0.114 ±0.01U/ml) and M_1 (0.21±0.06U/ml) in sodium chloride, potassium chloride, calcium chloride, magnesium chloride and zinc chloride as metal ion source,

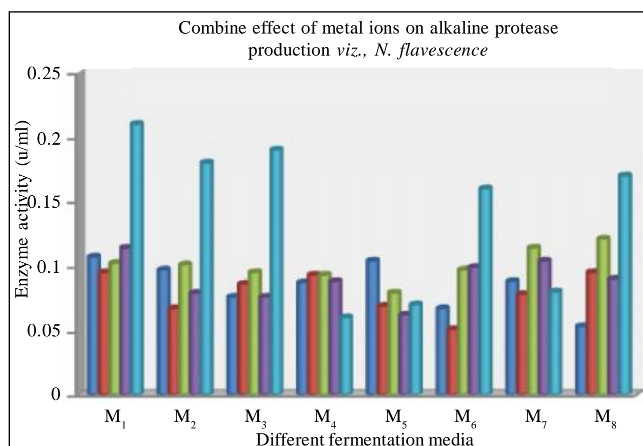

Fig. 9 : Optimization of alkaline protease production from *N. flavescence* in different nitrogen source (sodium chloride, potassium chloride, calcium chloride, magnesium chloride and zinc chloride) (a) Maximum yield was observed in M_1 *viz.,* zinc chloride (b) Minimum yield in M_6 and M_5 *viz.,* potassium chloride and zinc chloride. All the values are ANOVA significant at $p<0.05$

respectively. Among these highest activity was in zinc chloride in M_1 (0.21±0.06U/ml) media was found to be significant for alkaline protease production (Fig. 9).The activity was enhanced (four-fold) by Cu^{2+} ions indicating the presence of metallo-protease (Doddapaneni *et al.,* 2007).

Effect of inhibitors on alkaline protease production:

The maximum inhibition was observed in M_8 (0.088±0.01U/ml),M_4(0.67±0.26U/ml), M_4 (0.095±0.01U/ml), M_4 (0.081±0.01U/ml) and M_4 (0.083±0.01U/ml) in copper sulphate, EDTA, SDS, ferric nitrate and β-mercapto-ethanol as protease inhibitors, respectively. Among these highest inhibition was in copper sulphate in M_4 (0.67±0.26U/ml) media (Fig. 10). The activity of alkaline protease was inhibited by EDTA (Doddapaneni *et al.,* 2007).

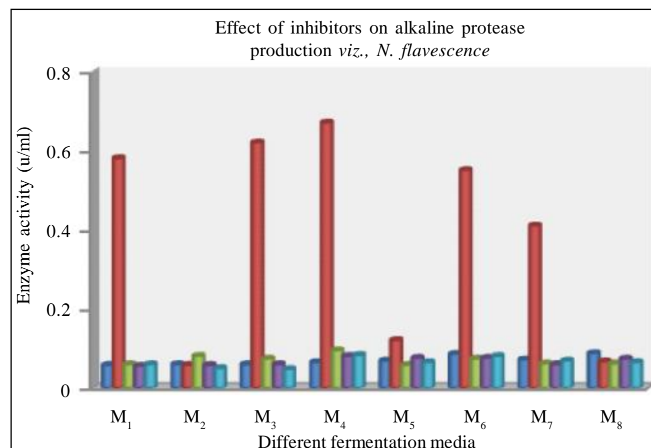

Fig. 10: Effect of inhibitors on alkaline protease production from *Neisseria flavescence* (copper sulphate, EDTA, SDS, ferric nitrate and -mercaptoethanol) (a) Maximum inhibition was observed in M_4 *viz.,* EDTA (b) Minimum inhibition in M_2 *viz.,* SDS. All the values are ANOVA significant at $p<0.05$

Effect of casein concentration on alkaline protease production isolated from *Neisseria flavescence* :

The effect of different concentration of substrate was studied on production of alkaline protease from *Neisseria flavescence.* The maximum production was observed in M_6 media at 1mg/ml of casein concentration (Fig. 11).

Compatibility of detergent :

Besides pH, a good detergent protease is expected to be stable in the presence of commercial detergents.

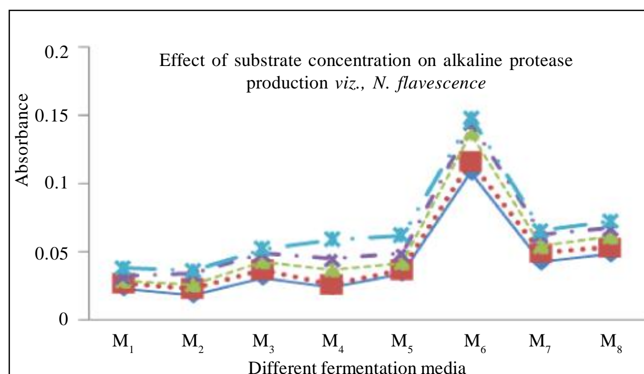

Fig.11 Effect of casein concentration on alkaline protease production; maximum production was observed in M_6 fermentation media. All the values are ANOVA significant at $p<0.05$

The protease showed excellent stability and compatibility in the presence of locally available detergents (Henko, Surf Excel, Tide and Rin). The extracted protease showed good stability and compatibility in the presence of Tide then Surf excel followed by Henko and Rin. The supplementation of the enzyme preparation in detergent (*i.e.,* Tide) was significantly improved the cleansing of the ink stains (Fig. 12 a, b and c).

Fig. 12: Washing performance of alkaline protease in the presence of detergent (Tide). (a) Cloth stained with ink; (b) ink-stained cloth washed with detergent only; and (c) ink-stained cloth washed with detergent and enzyme

Conclusion :

Microbial alkaline proteases are among the important hydrolytic enzyme and have been used extensively since the advent of enzymology. Bacterial extracellular alkaline proteases are of great importance due to its wide spectrum applications in detergent industries, bioremediation, food industries, and leather processing and bio-film degradation. From the various niches eighteen isolates were screened for alkaline protease production, out of which four isolates showed efficient alkaline protease

production. Out of four bacterial species one of the isolate *i.e., N. flavescence* showed significant enzyme activity. Optimization of the pH and temperature conditions for enzyme production was determined and was found to be 7.0 and 60^0C, respectively. Optimization of carbon, nitrogen sources and metal ions for enzyme production were determined and was found to be 4.64U/ml for sucrose, 0.91U/ml for gelatin and 0.21U/ml for zinc chloride for the isolates. The yield of alkaline protease was inhibited by copper sulphate. Enzyme activity was assayed using tyrosine-casein method. The purified enzyme preparations of having enzyme activity 0.26U/ml was also excellent in destaining of ink colour. The molecular weight of different bands of alkaline protease from *N. flavescence* ranged from 25kD to 83kD.

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