Production dynamics of extracellular alkaline protease from *Neisseria* sps. isolated from soil

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ABSTRACT

Proteases constitute one of the most important groups of industrial enzymes and have applications in different industries such as detergent, food, feed, pharmaceutical, leather, silk and for recovery of silver from used X-ray films. Soil samples collected from KMF milk processing plant situated at Dharwad, Karnataka were screened for alkaline protease producing bacteria on alkaline agar plates containing casein as substrate. Alkaline protease production was identified by clear zones of casein hydrolysis around colonies. Such colonies were grown in an alkaline broth for 72 h and the enzyme activity of the culture supernatants was determined by measuring the amount of tyrosine released from casein after 10 min at 35°C, at a pH of 10.5. The isolate with prominent zone of clearance was identified based on biochemical characterization, such as Gram staining, endospore, motility test, litmus milk reactions, carbohydrate fermentation, nitrate reduction, indole production, VP, citrate utilizations etc. and the isolate was identified as *Neisseria* sps. The physiochemical parameters of production medium were optimized for the isolate, *Bacillus subtilis* NCIM 2724 and *Pseudomonas aeruginosa*. The isolate produced protease at maximum rate at 48 h of incubation at 65°C with agitation speed of 60 in 10g/L of casein. The best carbon sources for this isolate was fructose after glucose. The partially purified alkaline protease at 55% ammonium sulphate saturation from the isolate showed reasonable total activity of 265.2 U with specific activity of 42.5 U/mg with purification fold of 6.97. Optimal temperature, pH, agitation speed, incubation period, carbon source and casein concentration of the proteases from *B. subtilis* NCIM 2724 were found to be at 65°C, 7.0, 140, 48h, fructose and mannose after glucose and 10g/L respectively. Partially purified alkaline protease gave total activity of 308.55 U with specific activity of 108.03 U/mg and purification fold of 10.84. The intracellular enzyme from *Pseudomonas aeruginosa* showed maximum activity around 50°C, at pH 8.0, 140 agitation speed at 60 h of incubation with lactose as best carbon source after glucose. The extracellular production of the enzyme, its thermostable nature, alkaline pH are features which suggest its application in the detergent industries.

Keywords: Alkaline protease, *Neisseria* sps., optimization, *Bacillus subtilis* NCIM 2724, *Pseudomonas aeruginosa*, ammonium sulphate

INTRODUCTION

As proteases are physiologically necessary for living organisms, they are ubiquitous, found in a wide diversity of sources such as plants, animals and microorganisms [1]. There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial community. Proteolytic enzymes reduced inflammation by neutralizing the biochemical of inflammation (i.e. bradykinins, proinflammatory and eicosanoids) to levels at which the synthesis, repair and regeneration of injured tissues can takes place [2]. Alkaline proteases are defined as those proteases which are active in a neutral to alkaline pH range [3]. The six specific catalytic types that are recognized are the serine, threonine, cysteine, aspartic acid, glutamic acid,
Alkaline proteases hydrolyze a peptide bond which has tyrosine, phenylalanine or leucine at the carboxyl side of the splitting bond. Alkaline serine proteases are the most important group of enzymes exploited commercially [5]. Proteases do not refer to a single enzyme but a mixture of enzymes [6]. These enzymes have become widely used in the detergent industry, since their introduction in 1914 as detergent additives.

However, until today, the largest share of the enzyme market has been held by detergent alkaline proteases active and stable in the alkaline pH range. They constitute 59% of the global market of industrial enzymes, which is expected to exceed $ 2.9 billion by 2012. Proteases are robust enzymes with considerable industrial potential in detergents, leather processing, silver recovery, medical purpose, food processing and also in waste water treatment [7]. Different companies worldwide have successfully launched several products based on alkaline proteases. The success of detergent enzymes has led to the discovery of a series of detergent proteases with specific uses [3]. The increased usage of these proteases as detergent additives is mainly due to the cleaning capabilities of these enzymes in environmentally acceptable detergents. 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 [8].

Proteases of bacteria, fungi and viruses are increasingly studied. Microorganisms represent an attractive source of proteases as they can be cultured in large quantities in a relatively short time by established fermentation methods producing an abundant, regular supply of the desired product. In general microbial proteases are extracellular in nature and are directly secreted into the fermentation broth by the producer, thus simplifying downstream processing of the enzyme as compared to proteases obtained from plants and animals [7]. In general, extracellular proteases catalyze the hydrolysis of large proteins to smaller molecules for subsequent absorption by the cell, whereas intracellular proteases play a critical role in the regulation of metabolism [9]. Bacteria which produce proteases include Bacillus sp., Alcaligens faecalis, Pseudomonas fluorescens and Aeromonas hydrophilia. The genus 'Bacillus' is an important source of industrial alkaline proteases and are probably the only genera being commercialized for alkaline protease production [10]. Bacillus subtilis is the main group that is used in international enzyme industry. Studies on other strains of Bacillus showed that nutritional, chemical and physical factors can influence protease production [11]. Pseudomonas aeruginosa was also reported to be the producer of intracellular alkaline proteases [12]. Aspergillus sps., Penicillium sp., Kluyveromyces marxianus, Nocardiosis sp., Thermoactinomyces vulgaris A60 and Thermomyces lanuginosus are the fungal sources which have been reported as proteases producers. Classical IgA proteases are secreted by Neisseria meningitidis, N. gonorrhoea, Haemophilus influenzae, and Streptococcus pneumoniae [13].

One of the most important characteristics that determine the industrial suitability of proteases is their requirement of high pH for optimum enzyme activity [14]. Among protease the subtilisin has been engineered for thermal and pH stability for use in laundry detergents [15]. Most of the alkaline proteases applied for industrial purposes face some limitations [16]. Despite the fact more than 3000 different enzymes have been identified and many of these have found their ways into biotechnological and industrial applications. The present toolbox is still not sufficient to meet all the demands [17]. Thermostable proteases are advantageous in some applications because higher processing temperatures can be employed, resulting in faster reaction rates, increase in solubility of nongaseous reactants and products, and reduced incidence of microbial contamination by mesophilic organisms [18]. However, the stability of enzyme at extreme pH is important in the study of protein structures and functions [19]. Extracellular protease production in microorganisms is also strongly influenced by media components. Protease synthesis is also affected by rapidly metabolizable carbon and nitrogen sources in the medium. Besides these, several other physical
factors such as aeration, inoculum size, pH, temperature and incubation, also affect the amount of protease produced [20,21].

Optimization of medium components which have been predicted to play a significant role in enhancing the production of alkaline proteases. Various sources of carbon such as glucose, fructose, sucrose, maltose, starch and cellobiose are employed and there is a differential enzyme production and growth rate utilizing different carbon sources. No defined medium has been established for the best production of alkaline proteases from different microbial sources [8]. Each organism or strain has its own special conditions for maximum enzyme production. Purification of proteases to homogeneity is a prerequisite for studying their mechanism of action. Vast numbers of purification procedures for proteases have been well documented [9].

Enzyme activity increases with many fold by employing chromatographic techniques. Being the most important sources for enzyme production, the selection of suitable microorganism plays a key role in high yield of desirable enzyme. Looking into the depth of microbial diversity, there is always a chance of finding microorganisms producing novel enzymes with better properties and suitable for commercial exploitation. Microbial diversity is a major resource for biotechnological products and processes. Recent approaches for increasing protease yield include isolation and screening for hyper-producing strains and optimization of the fermentation medium.

MATERIALS AND METHODS

Isolation and identification

Three different soil samples were collected from dairy contaminated sites of Karnataka Milk Federation (KMF), Dharwad. 5gm of each soil samples was diluted with distilled water (50ml), serially diluted in order to reduce the initial number of microorganisms. These dilutions were then inoculated into alkaline agar media containing casein as substrate. All incubations were done at 30ºC and incubated in controlled-environment. The isolated colonies were transferred to alkaline broth. Different dilutions of above broth were streaked on Skim milk agar plates for testing the caseinolytic activity of the organisms. Bacteria were inoculated onto plates and incubated at 37ºC for 24 h. Strains that produced clearing zones in this medium were selected [22]. Formation of halo zone around the colonies, resulting from casein hydrolysis, was taken as evidence of proteolytic activity. The colonies producing clearing zones by hydrolysis of skim milk was used as indication of an alkaline protease producer. The organism screened with casein agar plates were subcultured by continuously growing the bacterium in basal broth medium at 37ºC and subsequently streaking on basal agar medium (2% agar-agar, 2 days at 37ºC).

Eventually, after several weeks of repeated selection and subculturing a pure culture of the bacterium was obtained. The resulting isolated colonies were subcultured onto nutrient agar slants, grown at 37ºC for 24 h, maintained at 4ºC and subcultured at an interval of four-weeks. The bacterial isolate with prominent zones of clearance was processed for the determination of morphology, gram characteristics, motility, endospore, citrate utilization, nitrate reduction, methyl red test, urease, catalase, VP and indol tests, acid production from dextrose, lactose and sucrose, starch hydrolysis, lipid hydrolysis, hydrogen sulphide test and triple sugar iron agar. The isolate was then identified in accordance with the methods recommended [23]. Bacillus subtilis NCIM 2724 strain and Pseudomonas aeruginosa were used as a standard organism for reference.

Production of alkaline protease

Inoculum was prepared by inoculating one loopful of culture into 10 mL alkaline broth medium and incubated at 37ºC for 24 h on a rotary shaker at 60 rpm. The inoculum developed for all the three
organisms, *Bacillus subtilis* NCIM 2724, *Pseudomonas aeruginosa* and the isolated bacteria were added and grown in an alkaline broth medium (g/L) K$_2$HPO$_4$ (2), (NH$_4$)$_2$SO$_4$ (1.4), MgSO$_4$.7H$_2$O (0.3), urea (0.3), CaCl$_2$ (0.3), casein (10), glucose (2.5), yeast extract (0.5) in 250 ml Erlenmeyer flasks. The pH of medium was adjusted to 9 before autoclaving at 121ºC for 20 min. and incubated at 35ºC for 72 h with gentle shaking at 60 rpm. The samples were collected at regular intervals of time and the cultures were centrifuged at 10000 × g for 10 min at 4ºC. The cell-free supernatant was used as the source of crude alkaline protease enzyme for assay.

**Protease assay**

The protease activity of the culture supernatants were then determined by adding 0.5 ml of the supernatant to 3 ml of the reaction mixture (6% casein in 10 mM borax buffer, pH 10.5) and incubating the mixture at 35ºC for 10 min. The enzyme activity was stopped by adding 3.2 ml of a solution containing 0.11 M trichloroacetic acid, 0.22 M sodium acetate and 0.33M acetic acid. The mixture was then incubated at 35ºC for 10 additional min and filtered. Alkaline protease activity was determined by measuring the amount of tyrosine released from casein at 275 nm [24]. One enzyme unit was the amount of enzyme which produced 1µmoles of tyrosine at 35ºC after 10 min of incubation. Total protein contents of the enzyme solution were measured according to the method described by Lowry [25] using bovine serum albumin (BSA) as a standard.

**Physicochemical optimization of enzyme production**

Alkaline protease production was optimized for *Bacillus subtilis* NCIM 2724, *Pseudomonas aeruginosa* and the isolated bacteria. Optimization for all the three organisms were carried out by varying concentrations of substrate casein (0.5-1.0%) in the media with incubation period range of 12-72 hrs. The agitation speeds tested were at 60 and 140 rpm and the effect of temperature on protease production was studied by growing each organism in fermentation media set at different temperatures (30, 40, 50 and 55ºC). The effect of pH on protease production was determined by growing each organism in fermentation media of different pH using appropriate buffers, Tris-HCl buffer (pH 7.0-8.0) and glycine-NaOH buffer (pH 9-10).

The various carbon sources chosen for the study were fructose, sucrose, mannose, maltose and lactose. These carbon sources were used to replace the carbon source available in the media. For all the experiments, samples were harvested from the flasks aseptically between 12-72 h post-inoculation and processed as described previously and protease activity was measured under various experimental conditions (enzyme units/ml supernatant) and the total cell mass was determined by measuring the optical density of the cultures at 660 nm.

**Partial characterization of the crude enzyme activity**

The crude alkaline protease was prepared as described above. The influence of temperature on the catalytic activity of the crude alkaline protease was determined by measuring the enzyme activity at temperatures range from 15ºC to 75ºC under the standard assay conditions. The influence of pH on the crude alkaline protease activity was determined by measuring the enzyme activity at varying pH values ranging from 7 to 11 using different suitable buffers, 50 mM sodium phosphate buffer (pH 7.0 and 8.0) and 50 mM Glycine-NaOH buffer (pH 9.0, 10.0 and 11.0) respectively [26]. The effect of substrate concentration range of 30-120µg was studies for all the organisms. The Km and Vmax value of the proteases were determined for respective organisms as the initial velocity is equal to the amount of product formed per unit time [12].
Partial purification of enzyme

Purification of enzyme was carried out at lower temperature and the microorganisms were grown at 35°C for 2-4 days. The culture was centrifuged at 10000 g for 10 min at 4°C and the supernatant was retained as the source of extracellular enzyme. Ammonium sulphate saturation was carried out, to the cell free supernatant, solid ammonium sulphate (35-55% saturation) was added and centrifuged at 20000g for 20 mins at 4°C. After resuspension of precipitated phase in phosphate buffer, it was dialyzed for 24 h at 4°C against the same buffer (Cut off<10 kDa). The concentrated enzyme activity, protein content and specific activity was calculated.

RESULTS AND DISCUSSION

Isolation of alkaline protease producing bacteria was carried out using rich alkaline media containing casein as substrate and further the obtained isolates were screened on Skim milk agar plates. Formation of clear zone around colonies was considered as indication of alkaline protease production. Isolates showing large clear zone around their colonies were isolated. Out of 15 isolates showing halo zone around the colonies, one potential bacterial isolate was selected for further investigation and subjected for biochemical characterization. The isolate was a Gram negative, Diplococci, non-spore forming, non-motile, catalase positive, indole and VP negative. Carbohydrate fermentation showed positive for dextrose, sucrose and lactose. The bacterium was able to hydrolyze casein but not starch. It grew in media containing citrate and was able to utilize citrate, urease activity and lipid hydrolysis showed negative, nitrate reduction and methyl red test showed positive and no hydrogen sulphide production was observed for the isolate. Collectively, these biochemical characteristics indicated that the isolate belongs to the genus Neisseria [23].

It has been reported that the production of extracellular proteases by different microorganisms can be strongly influenced by the culture conditions [27]. 10g/L of casein concentration in the media gave good activity in all the organisms shown in figure 1. The incubation period of a fermentation experiment has a direct relationship with the growth of microorganism and production of enzymes. Figure 2 shows B. subtilis NCIM 2724 and Neisseria sps. at 48 h of incubation showed increased activity of 3.5U/ml and 3.2U/ml respectively. It was found that the maximum production of protease and bacterial biomass were achieved after 48 h of incubation, which corresponded to the logarithmic growth phase of the bacterium [28]. B. firmus MTCC 7728 studied showed that the enzyme activity was maximum after the isolate reached stationary phase around 48 h of growth [29]. It is clear from the results that the incubation period of more or less than 48 h did not show the promising results as far as production of the enzyme is concerned.

Other workers suggest that prolonged incubation time perhaps led to autodigestion of proteases by other proteases [30]. Enzyme production may occur at stationary phase hence long incubation hours are required. P. aeruginosa had 60 hrs in line with proteases reported from SSA1 where the enzyme production also started after 72 hrs of incubation, hence it was categorized as secondary metabolite [27]. Alkaline proteases production occurs in late stationary phase and increases as GTP levels decreases [5]. B. subtilis NCIM 2724 at 48 h of incubation with 140 agitaion speed showed highest activity of 4.47U/ml, P. aeruginosa at 60 h of incubation at 140 agitaion speed showed activity of 1.85 U/ml as shown in figure 2 and 3, which in agreement with Bacillus cereus strain 146 grown in culture media showed maximum protease activity at higher 170 agitaion speed after 48 h of incubation. Improved distribution of dissolve oxygen and more effective uptake of nutrient contributed to higher protease production [11]. At this speed, aeration of the culture medium was increased which could lead to sufficient supply of dissolved oxygen in the media [8]. The enzyme production by Neisseria sps. was good at 60 rpm with activity of 4.07U/ml. Similar aeration effect on protease production by B. licheniformis was observed when shaking
speed was low at (60 rpm) [24]. This was perhaps due to denaturation of enzymes caused by high agitation speed. Optimized agitation speed is necessary for protease production [31]. Based on these results further experiments were carried out with optimized rpm. *B. subtilis* NCIM 2724 at 50ºC showed good activity of 3.38U/ml and *Bacillus* species have shown maximum protease production at 60ºC [18]. *P. aeruginosa* at 50ºC showed activity of 1.62 U/ml and *Neisseria* sps. at 50ºC showed activity of 3.35U/ml as shown in figure 4. The effect of incubation temperature on the production of protease by *Lactobacillus* IH8 was studied and production of protease were achieved at an incubation temperature of 35ºC and the strain was found to be able to grow even at 50ºC.

![Figure 1. Effect of casein concentration on enzyme production.](image1)

![Figure 2. Effect of incubation period on alkaline protease production.](image2)

![Figure 3. Effect of agitation speed on alkaline protease production.](image3)
Environmental temperature is a very important parameter that is inescapably linked to the biomass production of an organism because cell temperature is always equal to the temperature of the culture medium. It affects the rates of cell reactions, the nature of the metabolism, the biomass composition and the nutritional requirements of the organism. Therefore, incubation temperature must always be considered an important parameter while carrying out the fermentation experiments [28]. *B. firmus* 7728 was reported for maximum proteolytic activity observed at pH 9 and temperature 40ºC [29]. Enzyme production in range of 30-50ºC and alkaline pH was optimum which was also found in other bacterial strains producing proteases [24]. In most studies on microbial cultures, the pH of the culture medium is one of the most important factors that influence the microbial growth and metabolism [28]. However, maximum protease production was observed at pH 8.0 for *B. subtilis* with activity of 3.30U/ml, *P. aeruginosa* with activity of 1.10 U/ml and *Neisseria* sps. with activity of 3.06U/ml as shown in figure 5. Similar results have been reported from many isolated bacterial strains [18]. Protease production by *Lactobacillus* was in contrast to our findings where the organism was very sensitive to pH change especially toward alkaline values because a slight increase in optimum pH resulted in much-reduced biomass and protease production [28].

It was suggested that sources of carbon affected production of enzymes by bacteria [32]. The various carbon sources such as fructose, sucrose, mannose, maltose and lactose were used in place of glucose which was the original carbon source in growth media. *B. subtilis* NCIM 2724 at 48 h of incubation showed activity of 2.21U/ml and 2.29U/ml with fructose and mannose respectively in comparison to glucose which showed highest activity of 4.47U/ml (Figure 6). Statistical significance in activity with carbon sources was not found. Significant improvement in protease production was observed with fructose and mannose.
yield was obtained with supplementation with maltose in *B. clausii* I-52 [16]. *P. aeruginosa* at 60 hrs of incubation showed good activity of 1.93 U/ml with lactose as carbon source. In addition to glucose, lactose has a significant positive effect on enzyme production in *Pseudomonas aeruginosa*, whereas other sugars showed slight activity (Figure 7). *Neisseria* sps. at 48 hrs of incubation showed slightly higher activity of 1.06 U/ml with fructose when compared to other sugars but ideally glucose had a better effect on enzyme production and significantly showed highest enzyme activity of 4.07U/ml (Figure 8). Results obtained showed that glucose instigated highest protease production compared to other carbon sources which is in good agreement with studies previously carried out [11]. Interestingly, for most of the isolates enzyme secretion was optimum with lactose [33]. Enzyme from *B. subtilis* NCIM 2724 and *Neisseria* sps. was stable upto 65°C and showed activity of 4.2U/ml and 3.60U/ml respectively whereas enzyme from *P. aeruginosa* was stable upto 55°C and showed activity of 1.25 U/ml as shown in figure 9. It was found that the crude alkaline protease of all the three organisms had a relatively wide pH range of activity between pH 8 to 10 as shown in figure 10, with maximum enzyme activity at pH 8.

![Figure 6. Effect of various carbon sources on alkaline protease production by *Bacillus subtilis* NCIM 2724.](image1)

Figure 6. Effect of various carbon sources on alkaline protease production by *Bacillus subtilis* NCIM 2724.

![Figure 7. Effect of various carbon sources on alkaline protease production by *P. aeruginosa*.](image2)

Figure 7. Effect of various carbon sources on alkaline protease production by *P. aeruginosa*.

Crude alkaline proteases from *B. halodurans* WN-SK5 also showed wide pH range of activity between pH 8 to 11 [26]. Higher temperature increased the rate of enzyme’s catalyzed reaction which resulted in increase of proteolytic activity as reported by *B. lichniformis* N-2 [17,26]. Others also reported proteases from *B. brevis* with these properties [27]. Having stability at high temperature and pH capability of our strains to secrete proteolytic enzymes under alkaline condition
was also reported by *Bacillus licheniformis* N-2 which in good agreement with our findings. Similarly, proteases from *Halogeometricum borinquense* strain TSS101 was stable over a broad pH range of 6.0-10.0 at 60ºC, indicating that it is an alkalophilic enzyme [34].

Figure 8. Effect of various carbon sources on alkaline protease production by isolate.

Figure 9. Effect of temperature on crude enzyme activity.

Figure 10. Effect of pH on crude enzyme activity.
Alkaline proteases from SSA1 also reported the broad pH range stability 7-13 [27]. pH around 11 and temperature of 60 and 65°C was recorded for *B. clausii* I-52 [16]. Protease activity was tested using different concentrations of casein as substrate and the Km and Vm values of the crude enzyme was determined by Lineweaver-Burk double reciprocal plot and was found to be Km=67µg, 40µg and 45.45µg for *B. subtilis* NCIM 2724, *P. aeruginosa* and *Neisseria* sps. respectively (Figure 11). The value of Vm=14.28 U/ml, 10 U/ml and 12.5 U/ml for *B. subtilis* NCIM 2724, *P. aeruginosa* and *Neisseria* sps. respectively. Similar results have been reported by others also [12]. Our finding are in line with other reports where protease activity obeyed Michaelis-Menten type kinetics when azocasein was used as substrate in *Bacillus amyloliquefaciens* S94.

![Figure 11. Effect of substrate concentration on crude enzyme activity.](image)

Kinetic parameter (Vmax and Km) values for the hydrolysis of azocasein by the protease were determined by plotting substrate concentration versus velocity [9]. Proteases from *B. subtilis* NCIM 2724 and *Neisseria* sps. was partially purified by ammonium sulphate precipitation and dialyzed, their total activity was found to be 308.55 U and 265.2 U respectively. With purification fold of 11 and 7 respectively at 55% saturation. The specific activity of the partially purified enzyme was 108.03 U/mg⁻¹ and 42.5 U/mg⁻¹ protein respectively for *B. subtilis* NCIM 2724 and *Neisseria* sps. Results of the purification of the protease secreted by *H. borinquense* strain TSS101 was reported by gel permeation column with high purification fold [34]. Proteases from SSA1 was also subjected to 80% partially purification by ammonium sulphate precipitation [27]. Enzyme from *Bacillus* sp. S17110 was purified from the culture supernatant and the enzyme was purified about 1.7 fold with total activity of 25.05 U concentrated by ultrafiltration and 70% ammonium sulfate precipitation [35]. The protease activity was maximally precipitated from the cleared growth medium [9]. *Stenotrophomonas maltophilia* strain S-1 also reported increase in specific activity at 80% ammonium sulphate saturation [36]. Proteases from *Bacillus* sp. 2-5 showed a purification fold of 29 by salting out [37]. In the present investigation we have determined the optimum parameters for maximum production of alkaline protease by the newly isolated bacterium *Neisseria* sps. and is quite promising as *Bacillus subtilis*, which are known for their ability to produce proteolytic enzymes with potential use in industries.

REFERENCES