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Thermostable Alkaline Protease from a Soil Isolate of Alkalophilic *Bacillus* species

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A soil isolate of *Bacillus* species (UVV1) was identified to produce extracellular alkaline protease under submerged fermentation in shake flask culture. Casein digestion was followed for assaying the alkaline protease activity. The operation variables optimized for improved alkaline protease production were pH, temperature and incubation time. A pH of 9 and temperature range of 50°C - 60°C favour the growth of the isolate as well as the production of protease with highest activity. Protease production was increased as the incubation time was prolonged up to 120 hours except for the span of 48 – 72 hours when a reduction in enzyme activity was noticed. The characterization studies on the crude enzyme reveal that the proteolytic activity was maximum at pH 9 and temperature 60° C.

Key words: Bacillus soil isolate, Alkaline protease, Casein digestion, Production optimization, Enzyme activity.

The use of alkaline proteases as industrial catalysts has been increasing in recent years as they find multifarious applications in detergent, protein, brewing, meat, dairy, photographic, and pharmaceutical industries [1, 2]. Owing to their selective peptide bond cleavage proteases are extensively used in the areas of structure elucidation whereas their synthetic capacities are used for the synthesis of proteins[2]. Concerning the sources of proteases, microbial proteases account for approximately 40% of the total worldwide enzyme sales. Although a wide range of microorganisms are known to produce proteases, alkalophilic Bacillus species are considered as prolific producers of alkaline proteases which exhibit significant activity, stability at high pH and temperatures and broad substrate specificity. The recent trend towards the use of Bacillus derived alkaline proteases in different process applications has increased remarkably due to their high production capacity and activity[3].

The technological application of alkaline proteases under demanding industrial conditions

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and molecular biological works makes the search for new microbial sources a continual exercise and they have prompted the isolation of alkalophilic microorganisms from a variety of natural and manmade alkaline environments[4]. Soil, with its tremendous microbial abundance and diversity, exists as an excellent source of new isolates producing enzymes of improved yield and activities at extreme conditions involved in many industrial applications. Consequently, we attempted to isolate a bacillus strain producing extracellular alkaline protease from soil samples collected from detergent industrial and agricultural areas. The study was further focused to optimize the culture conditions such as pH, temperature and incubation time using an optimized fermentation medium for the growth of the isolate in order to obtain maximum yields of alkaline protease.

MATERIAL AND METHODS

A new strain of *Bacillus* species (UVV1) isolated from the detergent industry waste soil in SIPCOT industrial estate, Madurai, Tamilnadu, India was initially characterized using morphological characteristics, standard biochemical tests and gram-staining techniques and further confirmed for protease production using starch casein hydrolysis test[5]. The strain was maintained on heart infusion agar medium and stored at 4°C.

For enzyme production, UVV1 was cultivated by shake flask culture method using yeast extract culture medium[6] containing g/L glucose 10; casein 5; yeast extract 5; potassium dihydrogen orthophosphate 2; sodium carbonate 10. Sodium carbonate solution was sterilized separately and added aseptically to the sterile medium and the initial pH of the medium was 9. The medium (100 ml) was inoculated with 1ml of freshly prepared bacterial suspension from a 3 day slant culture in 250 ml Erlenmeyer flask and incubated at 50°C with shaking at 200 rev min^{-1} on a rotary shaker for 120 h. The cell free supernatant was recovered by centrifugation (1000 rpm, 4°C, 15 min).Crude enzyme precipitated on addition of acetone to the supernatant was freeze dried and subjected to protein estimation by Lowry method[7] and assay of enzyme activity. Biomass was determined by drying the pellet obtained after centrifugation at 65°C for 24 h until it reached equilibrium weight.

Protease Assay[8]

Alkaline protease activity was determined using casein digestion by following the degraded products of casein in terms of L-tyrosine quantitatively. Casein was used as a substrate at a concentration of 0.2%. Enzyme diluent used contains 0.03 M cysteine hydrochloride and 0.006 M EDTA sodium. One unit of enzyme activity is defined as the amount of the enzyme liberating one µg of tyrosine at 37°C under the standard assay conditions from the standard casein substrate. Specific protease activity was calculated from protease activity and protein content of the precipitated enzyme fraction.

Optimisation of culture conditions for enzyme production

Optimization of environmental and fermentation parameters is essential as Each organism or strain has its own special conditions for maximum enzyme production[9]. The culture conditions pH, temperature, and incubation period were optimized for maximum enzyme production using yeast extract casein medium. Protease production was followed at different levels of pH (5-11) with 1 N HCl or 1 N NaOH, temperature (30°C - 60°C) and incubation time (24 – 120 hours).

Characterization of the crude protease enzyme

The crude protease obtained from UVV 1 with the highest proteolytic activity was further studied for preliminary characterization. The effect of pH on proteolytic activity was examined by determining the enzyme activity at different pH levels (5, 7, 9, 11, 13) at 30°C. The effect of temperature on enzyme activity was studied at 40°C, 50°C, 60°C, 70°C and 80°C at pH 7.

RESULTS AND DISCUSSION

Isolated microorganism

The organism isolated from the detergent industry waste soil was found to be gram positive, motile and rod shaped bacterium arranged in chains and can ferment dextrose and sucrose. It was also positive to Voges- Proskauer test and for the formation of acid in mannitol salt agar test

based on which the isolate was identified to be Bacillus subtilis [5]. The strain coded UVV1, when grown in starch casein agar medium showed clearing (Fig 1) thereby confirming the protease production capability of the isolated organism.

Production of alkaline protease

As the production of alkaline protease was reported to be maximum with wheat bran as the substrate, 1% wheat bran was also included in the cultivation medium[10]. The cultivation conditions fixed for the initial phase were pH 9, temperature 50°C and incubation time 120 h. The growth of the isolated bacterium at pH 9 reveals its alkalophilic nature. As *Bacillus* Sp. are specific producers of extracellular proteases[11], extracellular extract was separated by centrifugation after cultivation and the pellets were collected and weighed. Precipitation which is the most commonly used method for the recovery of protein from crude biological mixtures is generally effected by the addition of reagents such as salt or an organic solvent, which lowers the solubility of the desired proteins in an aqueous solution[12]. In the present study, protein content of the extracellular extract was precipitated with acetone, as many reports revealed the use of acetone up to 80%[6, 13-15]. The precipitate was freeze dried and weighed.

Table 1. Effect of pH on the biomass and the production of extracellular crude protease by UVV_1 at temperature 30°C for 48 h

pH	Biomass (g/L)	Protease activity (U/mg)	Specific activity (U/mg of protein)
5	5.5 ±0.7	175.4±19.6	665.3±29.0
7	16.3±0.8	668.2±87.8	1550.0±200.3
9	32.2±1.2	3168.2±143.9	663.9±402.8
11	28.3±0.8	2372.5±87.9	2617.5±262.7

Values are given as mean ± S. D (n=3)

Table 2. Effect of temperature on the biomass and the production of extracellular crude protease by UVV1 at pH 9 for 48 h

Temperature(°C)	Biomass (g/L)	Protease activity (U/mg)	Specific Activity (U/mg of protein)
30	32.2 ± 1.2	3168.2 ± 143.9	3663.9 ± 402.8
50	57.5 ± 0.9	5253.4 113.2	5450.9 383.5
70	30.0 ± 1.9	1957.2 ± 21.3	3587.4 ± 457.3

Values are given as mean ± S. D (n=3)

Table 3. Effect of Incubation time on Protease activity of crude enzyme precipitate from UVV_1 at pH 9, temperature 30°C

Incubation time (h)	Biomass (g/L)	Protease activity (U/mg)	Specific Activity (U/mg of protein)
24	5.1±0.6	43.9±10.0	876.05±18.5
48	32.2 ±1.2	3168.2±143.9	3663.9 ± 402.8
72	43.3±1.5	2092.8±50.5	2998.3±87.2
96	45.4±0.9	3868.9±231.9	4744.8±386.1
120	52.0±1.8	5598.0±54.9	6544.4±398.2

Optimization of cultivation variables

The important characteristic of most alkalophilic microorganisms is their strong dependence on the extracellular pH for cell growth and enzyme production. In addition the culture pH strongly affects many enzymatic proteases and transport of various components across the cell membrane[4]. The levels of pH were 5, 7, 9 and 11 and the temperature was maintained at 30°C and fermentation was continued for 48 h. Biomass obtained and protease activity of the extracellular crude enzyme portion are tabulated in Table 1.

The dry cell weight or the biomass weight was found to be maximum at pH 9 which was double the output at pH 7. As the pH was further increased to 11, the growth was found to be decreased indicated by reduced biomass but this was more than that at pH 7. These results clearly confirm the alkalophilic nature of the isolated organism. The activity of protease at pH 9 was found to be maximum which coincides with the higher growth (greater biomass). The specific activity was also found to be maximum at pH 9 but as the pH was further increased to 11, the specific activity was drastically decreased which may be due to a reduction in the total protein content of the crude precipitate at pH 11 compared to at 9 Thus the optimum pH for the production of protease is 9 which agrees with the earlier observations that the pH of the medium must be maintained above 7.5 throughout the fermentation period for increased protease yields from alkalophiles[16].

Temperature is another critical parameter that has to be controlled for maximum protease production as enzyme synthesis and energy metabolism in *Bacilli* are controlled by temperature and O_2 uptake[17]. The temperature levels chosen for present study were 30°C, 50°C and 70°C and the pH was maintained at 9 for all batches.

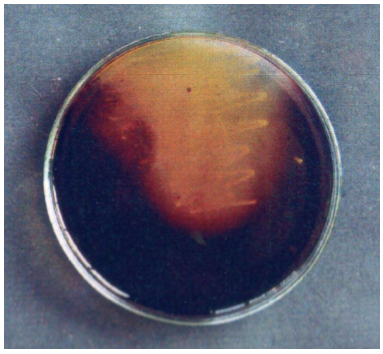

Fig. 1. Clearing zones in starch casein hydrolysis showing the protease production capability of UVV1

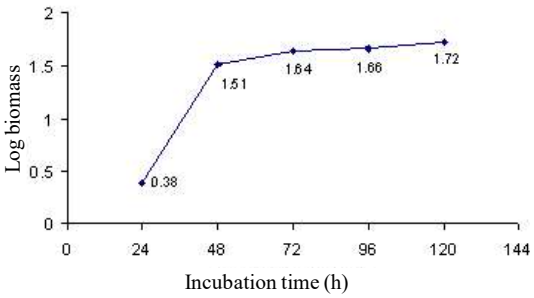

Fig. 2. Effect of incubation time on biomass

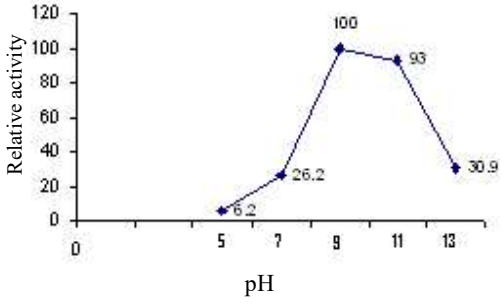

Fig. 3. Effect of pH on activity of alkaline protease produced by UVV1

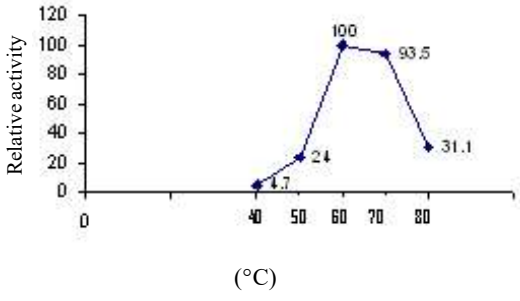

Fig. 4. Effect of temperature on activity of alkaline protease produced by UVV1

The results show that a temperature range of 50°C - 60°C is favourable to the growth of the isolated organism (UVV_1) indicated by greatest biomass, as well as maximum extracellular crude enzyme content and higher protease activity (Table 2). These results indicate the thermophilic character of the isolate, which corresponds to the previous finding that majority of the thermophilic *Bacilli* are found to grow in the temperature range of 50 - 65°C[10] and the optimum temperature for maximum protease production for different species of *Bacillus* may range from 30°C to 60°C[4].

The cultivations were done for different time periods 24 h, 48 h, 72 h, 96 h and 120 h maintaining the pH 9 and temperature 30°C in order to expose the correlation between incubation time and production of enzymes.UVV1 exhibits the transition state from exponential growth to the stationary phase 48 h as shown in the plot between log biomass and incubation time (Fig.2).

The enzyme production was found to increase upto 48 h after which a sudden reduction was observed at 72 hours. Again a steep rise in protease activity was noticed after 72 h that was sustained upto 120 h (Table 3). Under most growth conditions, *Bacillus* spp. produced extracellular proteinases during the post exponential phase[18]. The fluctuation in the production with respect to incubation time may be substantiated by the previous findings that the synthesis of protease in *Bacillus* species is constitutive or partially inducible and is controlled by numerous complex mechanisms and operative during the transition state between exponential growth and the stationary phase [10,19]. The reduction in protease production between 48 - 72 h may be attributed to the auto-degradative process [20].

Characterisation of enzyme activity at various pH and temperature

The effect of pH on activity was elucidated by carrying out the digestion of casein by the produced protease at different pH levels 5, 7, 9, 11 and 13 using 0.1N HCl or 1N NaOH at 25°C. The effect of temperature was determined by incubating the reaction mixture pH 7 for 1 min at different temperatures ranging from 40°C, 50°C, 60°C, 70°C and 80°C. The relative activity was calculated as the ratio of the proteolytic activity obtained to the maximum activity obtained at the range tested and expressed as percentages (Fig. 3, 4).

The enzyme was active over a wide pH range between 5-13, the maximum being noticed at pH 9-11.Also the alkaline protease produced is thermostable, which retained its activity even at high temperatures, the maximum activity being observed at 60°C.

This study which was conducted on an isolated *Bacillus* strain from soil, producing alkaline proteases has turned out to be fruitful in the respect of high degree of alkaline protease production and also the protease produced has been proved to be active in extreme experimental conditions prevailing in actual industrial and pharmaceutical applications.

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