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RESEARCH ARTICLE

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OPTIMIZATION OF CULTURAL CONDITIONS FOR THE OVER PRODUCTION OF ALKALINE PROTEASE FROM A MUTANT BACILLUS LICHENIFORMIS Bl8

ABSTRACT

Various factors influencing the alkaline protease production by mutant *Bacillus licheniformis* Bl8 were studied. The production was maximum when the culture was in the early stationary phase. The optimum temperature of incubation and the optimum initial pH requirement was 40^0C and pH 10.0 & 10.5 respectively. The incubation period required for the maximum accumulation of alkaline protease was 72h. The optimum level of inoculum of 10% was found to be favoring the production. The age of inoculum was found to be 24h for maximum protease productivity. Further increase in the age of inoculum was found to be having little (or) no effect on alkaline protease production. The best carbon source for the production was maltose and Yeast extract plus peptone was the best nitrogen sources. Casein was also found to increase the alkaline protease production. Effect of some other factors like vitamins, amino acids, trace elements, metabolic inhibitors, antibiotics, surfactants and agitation were studied. Among the various vitamins tested pyridoxine HCl was found to slightly stimulate the production whereas, other vitamins had little (or) no effect. Among the amino acids L-Tyrosine had shown the maximum increase. Among the all the trace elements tested, ammonium chloride augmented the maximum alkaline protease production. Different metabolic inhibitors viz., $AgNo_3$, EDTA, NaF, $KMnO_4$, β-ME, PMSF and Idoacetate were studied for their effect on alkaline protease production by *Bacillus licheniformis* (Bl8). All the inhibitors were showed the inhibitory effect on the protease production. Significantly PMSF showed complete loss of production revealings that the type of protease in the present study belongs to serine protease. And all the antibiotics tested were also showed inhibitory effect on alkaline protease production by the mutant Bl8 strain. This was also followed by the surfactants tested in this study. Agitation was found to be increasing the production significantly and the maximum yield observed at 150 rpm.

KEY WORDS Alkaline protease, mutant, *Bacillus licheniformis* Bl8, optimization

INTRODUCTION

Proteases are the single class of enzymes which occupy a pivotal position with respect to their application in both physiological and commercial fields and account for about 60% of the total worldwide sale of enzymes. Proteases are useful in the field of medicine also where they have some diagnostic and therapeutic applications (27). Among the different types of microbial proteases the most commercially important are the alkaline proteases, especially those from the bacterial sources. The commercial superiority of alkaline proteases is due to their suitability for use in the field of detergent industry, The suitable strain for production has been selected, mutation induction in that strain has been exploited to improve enzyme production. Now with the improved strain it is necessary to optimize the cultural conditions for maximum enzyme production so as to reduce the cost of production. The present study was carried out considerably by adjusting the nutritional parameters such as carbon sources and nitrogen sources along with physical factors like inoculum concentration, temperature, pH and incubation time. All of these factors can significantly affected alkaline protease production.

MATERIALS AND METHODS

With the objective of obtaining high yield of alkaline proteases, the factors influencing the production by the selected strain were studied. The mutant strain selected for the production by submerged fermentation was *Bacillus licheniformis* (Bl8). The factors influencing the production was studied, examining one factor at a time, keeping the other factor constant. Once the optimization has been done with respect to a factor it was incorporated in the experiment for the optimization of the next factor. Experiments were done in duplicate. The data presented here was mean of the experiments

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Microorganism

The bacterial strain of *Bacillus licheniformis* Bl8 isolated from alkaline soil of the milk processing unit and was mutated by UV irradiation (16).

Growth kinetics

The test organism was grown in Erlenmeyer flasks with Glucose, Yeast extract, Peptone medium (15) containing (g/l) Glucose 10, Yeast extract 5, Peptone 5, $MgSO_4$ 7.H_2O 0.2, K_2H PO_4 1, Distilled water 1000 ml and pH 9.5 inoculated with 10%(v/v) of 24h old seed culture prepared in the same medium incubated at 40^0C on incubatory shaker for for 120 h in an orbital shaker at 70rpm. Five ml of medium was withdrawn at regular interval at 12h and its absorbance was measured at 610nm. The contents were then centrifuged at 10,000 rpm for 20min the supernatant was used as the crude enzyme for the assay of alkaline protease activity, which was expressed in Uml^{-1}.

Enzyme assay

According to Udandi Boominadhan et al., (2009), the enzyme was assayed in the reaction mixture containing 2.0 ml of 0.5% casein solution in 0.1M Carbonate – Bicarbonate buffer pH 9.5 and 1ml enzyme solution in a total volume of 3.0ml. Reaction mixture was incubated for 5 min at 40^0C. The reaction was terminated by adding 3ml of 10% ice-cold trichloroacetic acid. The tubes were incubated for one hour at room temperature. Precipitate was filtered thorough whatman no.1 filter paper and the filtrate was collected. For the color development for the assay of tyrosine in the filtrate, 5ml of 0.4 M Sodium carbonate and 0.5 ml of Folin phenol reagent were added to 1ml of filtrate vortexed immediately and incubated for 20 min at room temperature optical density was taken at 660 nm. Concentration of tyrosine in the filtrate was read from a standard curve for tyrosine already prepared.

Effect of various initial pH values on alkaline protease production

The production medium was adjusted at various levels of pH (7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11.0, 11.5, 12.0) and incubated . The enzyme activity was determined using the procedure described earlier

Effect of various incubation temperatures on alkaline protease production

Flasks with the production medium were inoculated and incubated at various temperatures such as 30^0C, 35^0C, 40^0C, 45^0C, 50^0C, 55^0C and 60^0C for 48 h and the enzyme activity was determined

Effect of level of inoculum on alkaline protease production

Experiments were carried out using 4.0%, 6.0%, 8%, 10%, 12% and 15% inoculum

Effect of age of inoculum on alkaline protease production

Fermentation experiments were carried out using cultures of different age (12, 24, 36, 48, 60 and 72 hrs old culture as inoculum).

Effect of different carbon sources on alkaline protease production

Influence of various carbon sources on enzyme production was investigated by replacing glucose with different carbon sources at concentration of 1% in the medium. The carbon sources included were Galactose, Maltose, Mannose, Ribose, Mannitol, Glycerol, Fructose, Starch, Sucrose and Lactose

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Effect of nitrogen sources on alkaline protease production

Nitrogen sources tested were casein, yeast extract plus beef extract, Soya meal, gelatin, urea, soybean meal plus casein, malt extract. peptone, KNO3, $(NH_4)_2SO_4$, $NaNO_3$, yeast extract plus casein, beef extract and yeast extract.

Effect of various vitamins on alkaline protease production

Vitamin solutions were prepared by adding the specific amounts of vitamins to distilled water, sterilized by filtration and then added aseptically to the sterile basal medium at concentrations of 5µg/ml. A control was run with water instead of vitamins. The following vitamins viz., Riboflavin, Ascorbic acid, Biotin, Pyridoxine Hcl, and Folic acids were used.

Effect of amino acids on alkaline protease production

The effect of different amino acids like L-phenyl alanine, L-lysine, L-leucine, L-histidine, L-tyrosine, L-tryptophan, L-aspargine, glycine, B-alanine and cysteine on protease production was studied. The solutions of individual amino acids were prepared in distilled water. Sterilized by filtration and added aseptically to the sterile production medium at a concentration of 0.5%. A control was run using water.

Effect of trace elements on alkaline protease production

The effect of trace elements sodium molybdate, $ZnCl_2$, $CoCl_2$, $CdCl_2$, $HgCl_2$, $CaCl_2$, NaCl, $MnCl_2$, NH_4Cl, $CuCl_2$, $FeCl_3$ on the production of alkaline protease by B.licheniformis mutant Bl8was studied at a concentration of 0.04%

Effect of metabolic inhibitors on alkaline protease production

Experiments were carried out using different inhibitors ($AgNo_3$, (silver Nitrate), EDTA (Ethylene Diamine Tetra Acetate), NaF (Sodium fluoride) $KMnO_4$ (Potassium Permanganate), β-ME (β-mercapto ethanol), PMSF (Phenyl methyl sulfonyl fluoride), idoacetate at a concentration of 0.05% and the enzyme activities were assayed..

Effect of antibiotics on alkaline protease production

Experiments were carried out using solution of antibiotic substances Penicillin, streptomycin, neomycin, framycitin, cephalosporin and chloremphenocol were prepared aseptically in sterilized distilled water and added aseptically to the sterilized production medium at a concentration of 50 µg/ml

Effect of surfactants on alkaline protease production

Experiments were carried out using the production medium with various surfactants Tween 80, Sodium sulphate, H_2O_2, Acetamide, Arial, Surf-excel at a concentration of 0.04%

Effect of agitation on alkaline protease production

Effect of agitation on alkaline protease production by unagitated culture and the culture agitated at different levels viz., 70, 90, 110, 130, 150 and 170 rpm was determined

RESULTS AND DISCUSSION

Protease production of mutant *Bacillus licheniformis* Bl8 cultivated in 50ml GYP medium over 120 period increased exponentially from 36h reaching the maximum yield of 180 Uml^{-1} at the 72h of cultivation (fig 1). The growth was almost stationary between 72 and 84 h after which it started to decline. This suggests that production of protease by this strain started at early exponential phase and reaches maximum at the end of exponential phase that is early stationary phase. Different Bacillus species have been reported to be producing the maximum enzyme during the late exponential (2), post exponential (27,18) and stationary (23) phases of growth. The exact reason for the increased production of protease during the later stages of growth is not known. A coincidence of reaching of extracellular protease

production at the maximum level with sporulation, the event occurring mainly during the later stages of growth has been reported by some workers (25). The possibility for the existence of a relationship between the triggering of protease production and sporulation was observed in Bacillus spp (5). cannot be ruled out in this case also.

The organism has produced reasonable amounts of protease under neutral and highly alkaline conditions with highest yield 190 Uml^{-1} and pH 10.0 and 10.5 (fig 2). So the optimum pH for the protease production was found to be 10 (or) 10.5. It is clear that the organism grew well at a wide range of pH from7.0 -12.0. The maximum production was seen between 9.0 and 11.0. Alkaline protease production using media with alkaline pH was reported by various researchers. (10,4,23), Optimum pH for *Bacillus licheniformis* and *Bacillus coagulans* proteases was 10 (8).

The organism grew over a wide range of temperatures (30^0 to 60^0C), the maximum enzyme activity namely 190 Uml^{-1} was found to be at 40^0C followed by 180 Uml^{-1} at 45^0C and 170Uml-1 at 50^0C (fig 3). Increase in incubation temperature to 60^0C decreased the yield to 100 Uml^{-1}. Hence the optimum incubation temperature for protease production by this organism is 40^0C. However, this strain was also able to produce good yield at 45^0C and 50^0C. Temperatures at (or) around 45^0C have been reported to be optimum for the production of protease by bacteria such as *Bacillus* sp. P-001A (2) and *Bacillus licheniformis* S40 (24).

The organism grew well in the medium and maximum protease production 240 Uml^{-1} was achieved at 72h (fig 4). After that the protease production decreased gradually with increase in incubation periods. The incubation period for the production of alkaline protease was found to vary with different microorganisms (21,3,22). The optimum incubation time for enzyme production was 96h in *Bacillus licheniformis* and *B. coagulans* (1)

Maximum production of alkaline protease by this organism was 242 Uml^{-1} at the level of 10% and also observed that further increase in inoculum level did not increase the protease production (fig 5). The optimum level of inoculum for the alkaline protease production by *Bacillus* sp.K25 was about 1-8% and 12 % also was found to be satisfactory (11).

Culture of 24h age had maximum protease productivity i.e 240Uml^{-1} (fig 6) . Further increase in age of inoculum was found to have little or no effect on alkaline protease production by mutant *Bacillus licheniformis* (Bl8).Studies showed that the alkaline protease production by *Bacillus* sp.K25 was independent of the age of inoculums (11,20).

The results of the effect of the different carbon sources on alkaline protease production was revealed that maltose is the best carbon source followed by mannose, Glucose, fructose and sucrose (fig 7). Improved yields in the presence of lactose, maltose, sucrose and fructose have been noticed in different protease producing microbial species (24,13).

All the nitrogen sources were found to induce the alkaline protease production. The highest production of 290$Uml^{-1}$was obtained with the combination of yeast extract plus peptone and also with casein alone (fig 8). Also relatively best activity was observed with the combination yeast extract plus casein. A similar effect of the combination of this nitrogen source on protease production by other *Bacillus* spp has been reported (14). Results show that the organic nitrogen sources are better than the inorganic ones for the alkaline protease production. This observation is in conformity with the earlier reports on the repressing effect of inorganic nitrogen sources on bacterial alkaline protease production (9,24). The inorganic nitrogen sources are generally known to be repressing the production especially when used in higher concentrations (23). The inducing effect of organic nitrogen sources on bacterial alkaline protease production has been reported (7,12). Maltose and either yeast extract plus peptone (or) casein found to be the best carbon and nitrogen sources respectively, for the production of alkaline protease by *Bacillus licheniformis* (Bl8).

Among the various vitamins tested Pyridoxine HCl was able to stimulate the production of protease slightly up to 295 Uml^{-1} (fig 9). Other

vitamins did not stimulate the protease production compared to control. Similar observation was made for *Thermoactinomyces thalophilus* PEE14 for production of alkaline proteases (6).

Many of the amino acids (at 5µg/ml concentration) were shown to stimulate protease production by Bacillus licheniformis (Bl8) (fig 10). Among them L- tyrosine had shown maximum increase up to 650 Uml^{-1} protease production. Stimulating effect of other amino acids is in the order: L-tryptophan (580Uml^{-1}) > L-Lysine (520 Uml^{-1}) > L-Histidine (460 Uml^{-1})> Glycine and B-alanine (420 Uml^{-1}). Maximum alkaline protease activity produced by Bacillus spp was achieved in the presence of combinations of various nitrogen sources (14). L-leucine, L-cysteine, L-tyrosine, L- tryptophan, L-lysine, L-alanine, L-arginine, and L-aspargine increased the alkaline protease production in *Thermactinomyces thalophilus* PEE14 (6). Lysine stimulated the alkaline protease production up to 1% concentration in actinomycete (19).

The effect of various trace elements on alkaline protease production was studied (fig 11). The results revealed that $ZnCl_2$ and NH_4Cl showed stimulatory effect on alkaline protease production up to 360Uml^{-1} and 440Uml^{-1} respectively, where as sodium molybdate (110 Uml^{-1}) $CoCl_2$ (160Uml^{-1}), NaCl (170Uml^{-1}), $MnCl_2$(70Uml^{-1}) $CuCl_2$ (190 Uml^{-1}) $FeCl_3$ (220Uml^{-1}) showed inhibitory effect at the concentration 0.04% used in the experiment. Low concentrations of inorganic nitrogen sources have been reported to be promoting good production of the enzyme in some bacteria (7,9). The inorganic nitrogen sources are generally known to be repressing the production especially when used in higher concentrations (17,19). Sodium molybdate. Sodium tungstate, Strontium chloride and Ferrous phosphates exerted stimulatory effect on enzyme production in *Thermoactinomyces thalophilus* PEE 14 (6).

The Effect of different metabolic inhibitors was studied (Fig 12). It is evident from the results that all the inhibitors used inhibited protease production at 0.05% level. Protease production was strongly inhibited by PMSF (00 Uml^{-1}). Similar results were reported in *Thermoactinomyces thalophilus* PEE 14 (6). This investigation surprisingly revealed that the type of alkaline protease in this study belongs to serine protease because its production by *B.licheniformis* (Bl8) and its activity are totally inhibited by PMSF. It is characterized by serine group at their active site. Generally alkaline serine proteases are known to be subtilisins. Subtilisin Carlsberg produced by *Bacillus licheniformis* was discovered in 1947 by Linderstrom, Lang and O Hensen at the Carlsberg laboratory. It is widely used in detergents.

All antibiotics exerted inhibitory effect on protease production at the concentration (50µg/ml) used (fig 13). Framycitin (180Uml^{-1}) and Neomycin (200 Uml^{-1}) have highly inhibited the enzyme production than Penicillin (230 Uml^{-1}), Streptomycin (230Uml^{-1}), Cephalosporin (230 Uml^{-1}) and Chloremphenacol (230 Uml^{-1}).Similar reports were made in describing the effect of various antibiotics on protease production by *Thermoactinomyces thalophilus* PEE 14 (6).

All the surfactants inhibited protease production at 0.04% concentration (fig14). Surf excel (160Uml^{-1}), Sodium lauryl sulphate (190 Uml^{-1}) and Ariel (190Uml^{-1}) showed the highest inhibitory activity than Tween 80 and Tween 20. Similar inhibitory effects of surfactants were reported on alkaline protease production by *Thermoactionomyces thalophilus* PEE14 (6).

Unagitated culture alkaline protease was produced only in very low level (110 Uml^{-1}) (fig 15). The unagitated culture of this bacterium was characterized by diminished cell growth due to pellicle formation over the surface of the culture. The culture showed an increase in production with the increase in the agitation rate up to 150 rpm with a maximum production of 420 Uml^{-1}. Further agitation decreased the alkaline protease production to about 380 Uml^{-1}. Similar observations have been made on the submerged fermentation system for the production of alkaline protease by *Bacillus licheniformis* S40 (24*), Bacillus* sp (26) and *Bacillus* sp IS-3 (23). So the optimum agitation rate obtained in the present study for the production of alkaline protease was 150 rpm.

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CONCLUSION

The results of the present studies on bacterial alkaline proteases are of great relevance from an industrial viewpoint. The yield of alkaline protease was found very high with genetic modification by U.V mutation and also thermo stable and alkali stable in nature and hence can be used for the large scale production. Yeast extract plus peptone and maltose, which are cheap and easily available, can be used in submerged fermentation. This indicates the feasibility of economical production of alkaline protease using this strain *Bacillus licheniformis* (Bl8).

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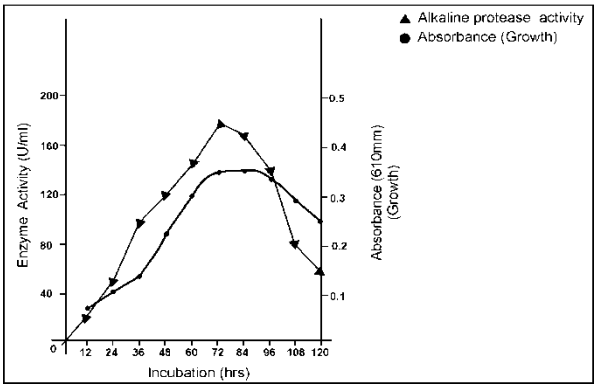

Fig 1 Growth kinetics of mutant Bl8 of Bacillus licheniformis and its enzyme production

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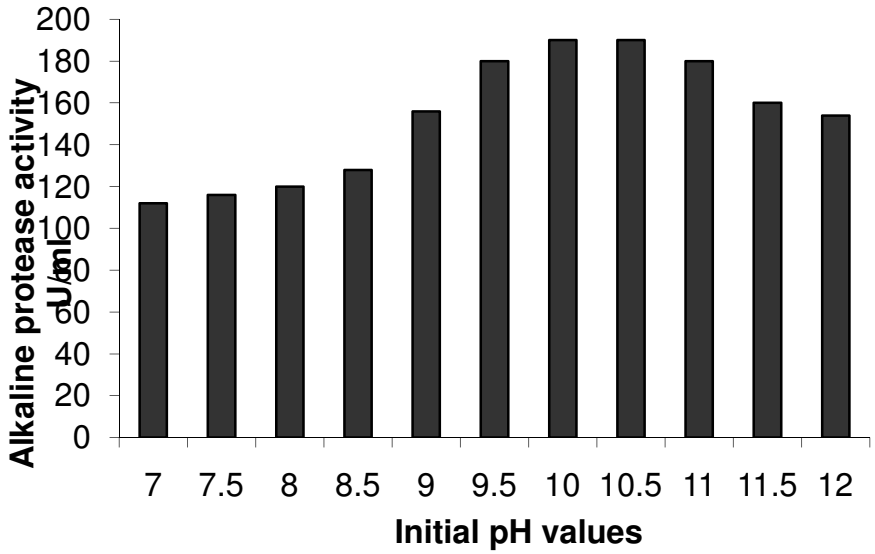

Fig 2 Effect of various initial pH values on alkaline **protease production by mutant *Bacillus licheniformis* (Bl8)**

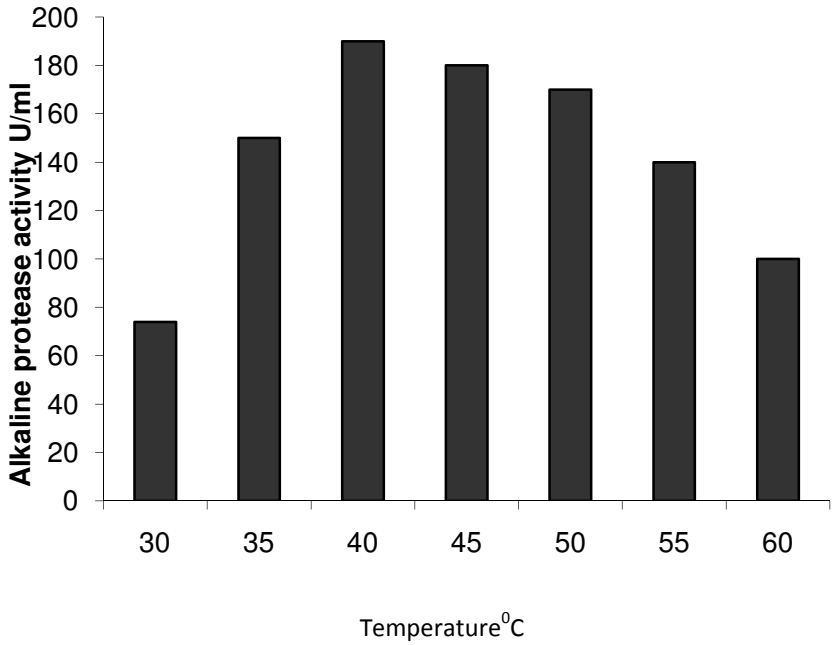

Fig 3 Effect of various incubation temperatures on alkaline protease production by mutant *Bacillus licheniformis* (Bl8)

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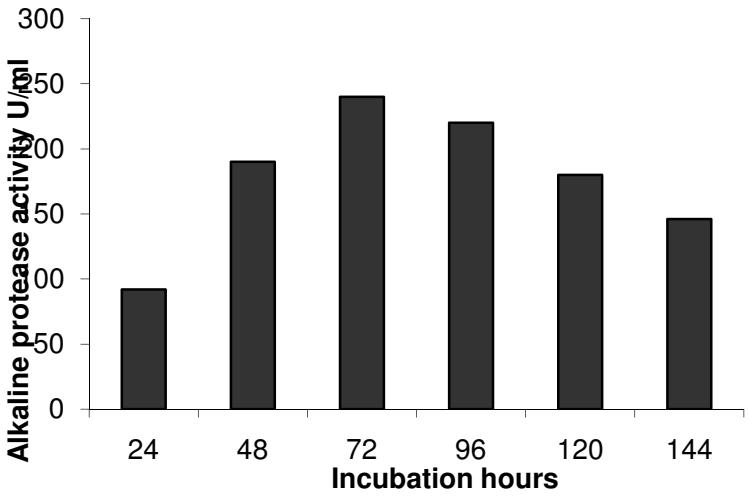

Fig 4 Effect of incubation period on alkaline protease production by mutant *Bacillus licheniformis* **(Bl8)**

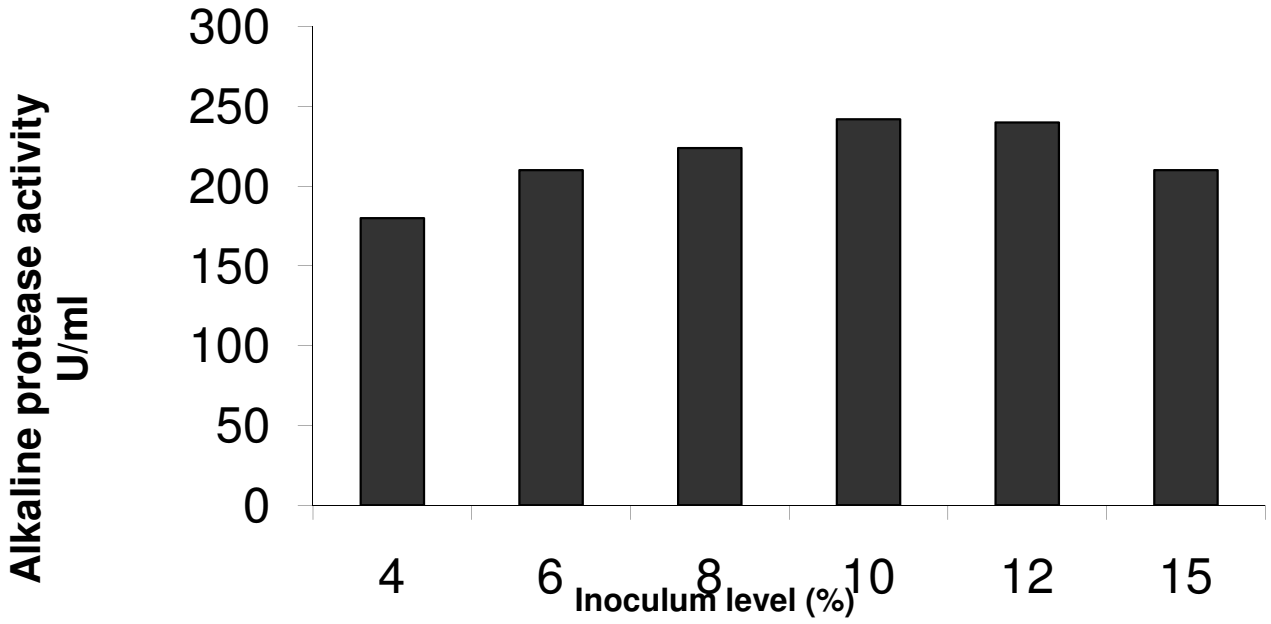

Fig.5 Effect of inoculum level on alkaline protease production by mutant *B.licheniformis* (Bl8)

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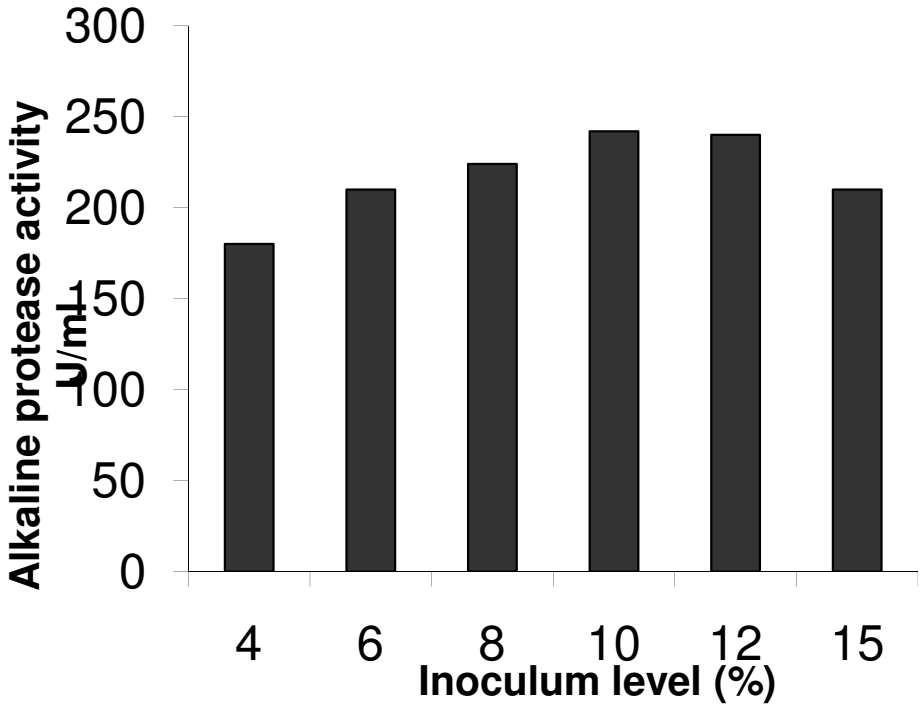

Fig.6 Effect of inoculums level on alkaline production by mutant B.Lichniformis (B18)

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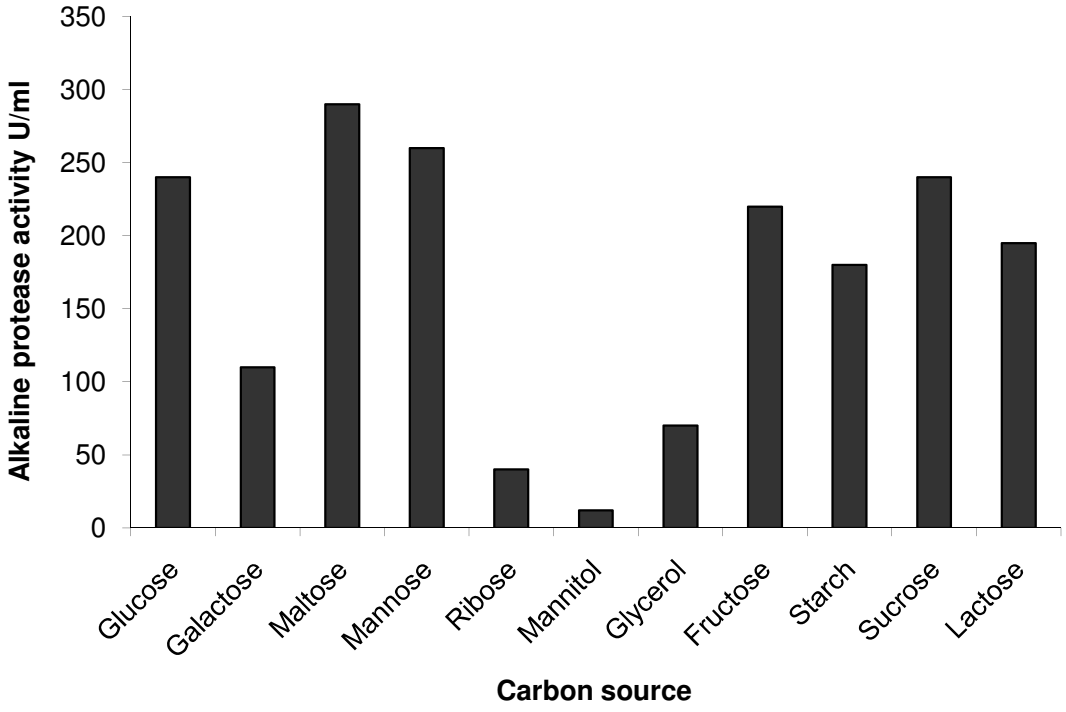

Fig.7 Effect of different carbon sources on alkaline protease production by mutant Bacillus licheniformis (B18)

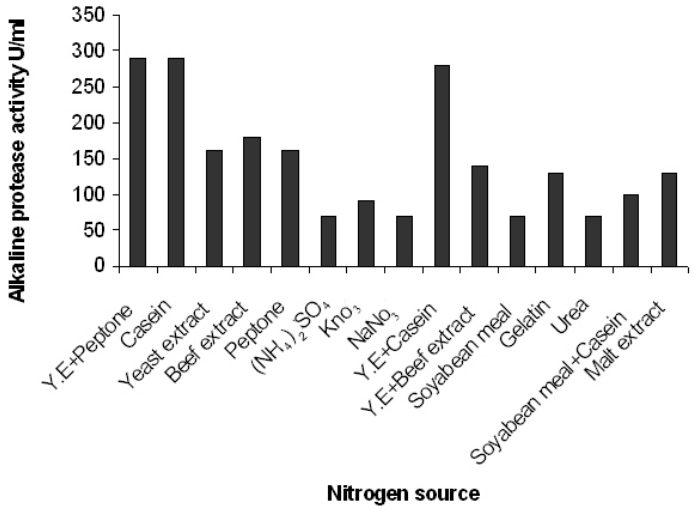

Fig 8 Effect of different nitrogen sources on alkaline protease production by mutant *Bacillus licheniformis* (Bl8)

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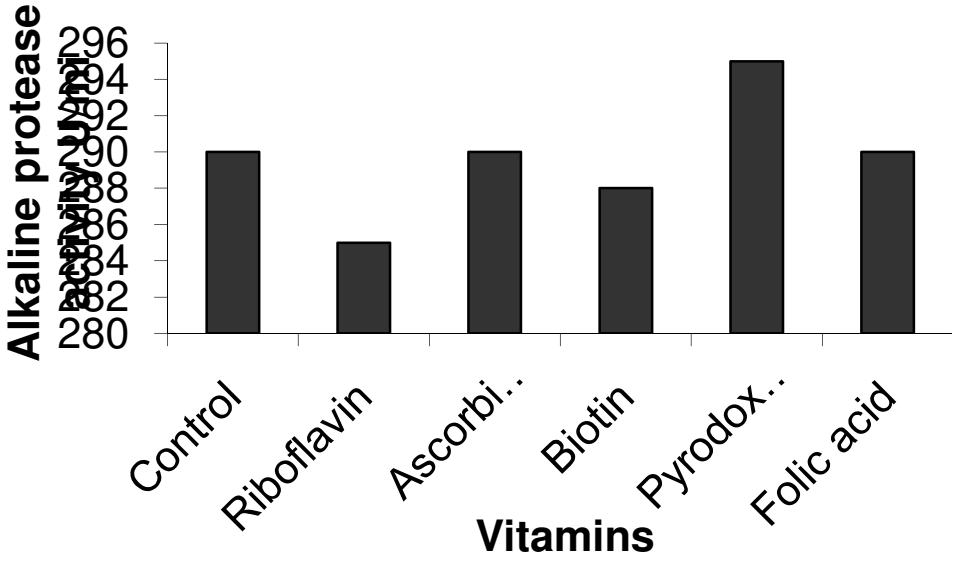

Fig 9 Effect of vitamins on alkaline protease production by mutant *Bacillus licheniformis* (Bl8)

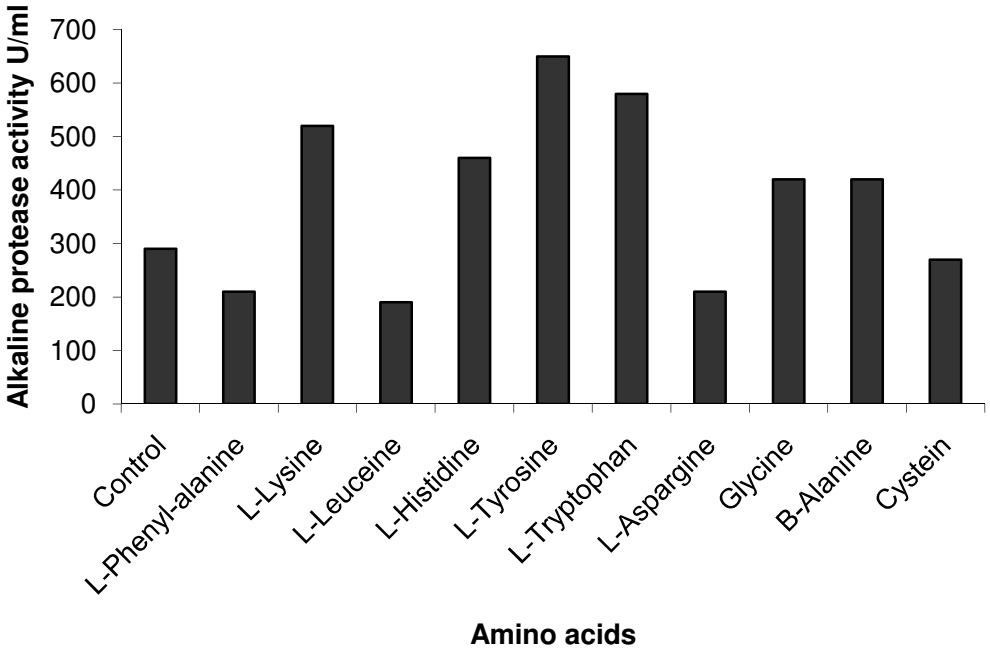

Fig 10 Effect of amino acids on alkaline protease production by mutant *Bacillus licheniformis* (Bl8)

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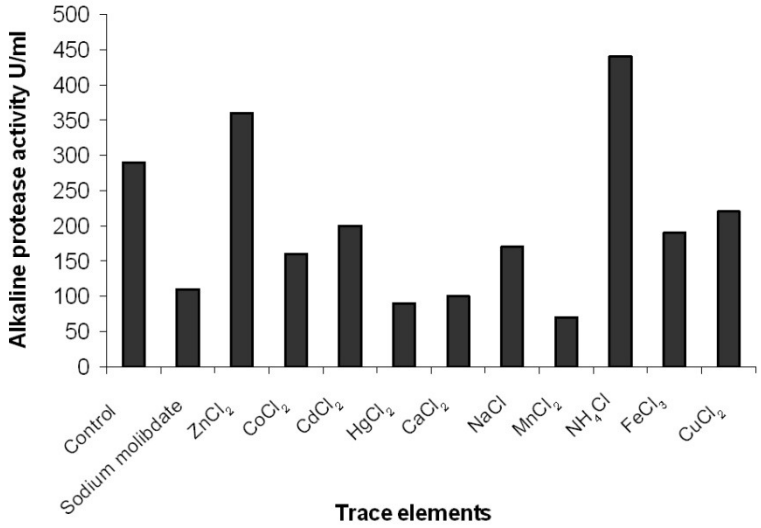

Fig 11 Effect of trace elements on alkaline protease production by mutant *Bacillus licheniformis* (Bl8)

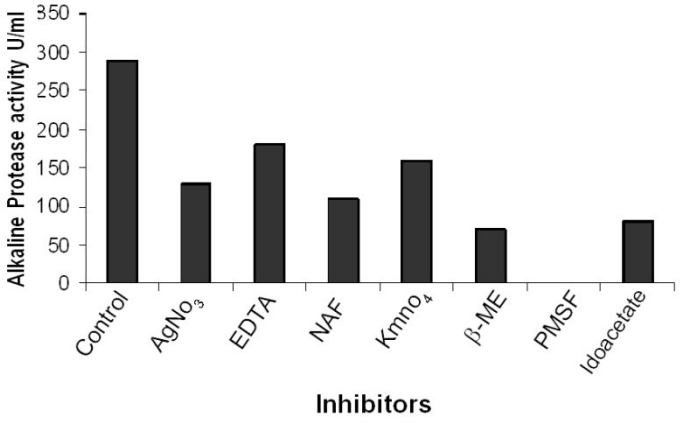

Fig 12 Effect of metabolic inhibitors on alkaline protease production by mutant *Bacillus licheniformis* (Bl8)

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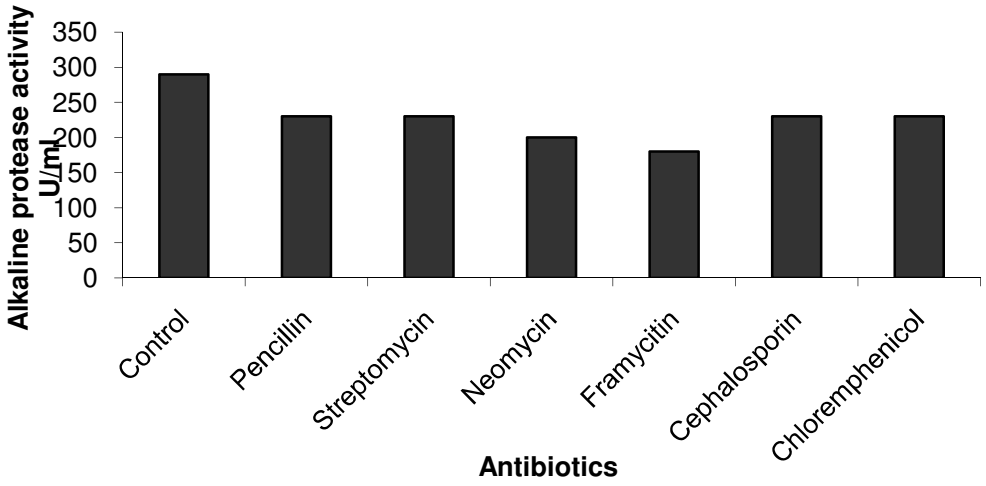

Fig 13 Effect of antibiotics on alkaline protease production by mutant *Bacillus licheniformis* (Bl8)

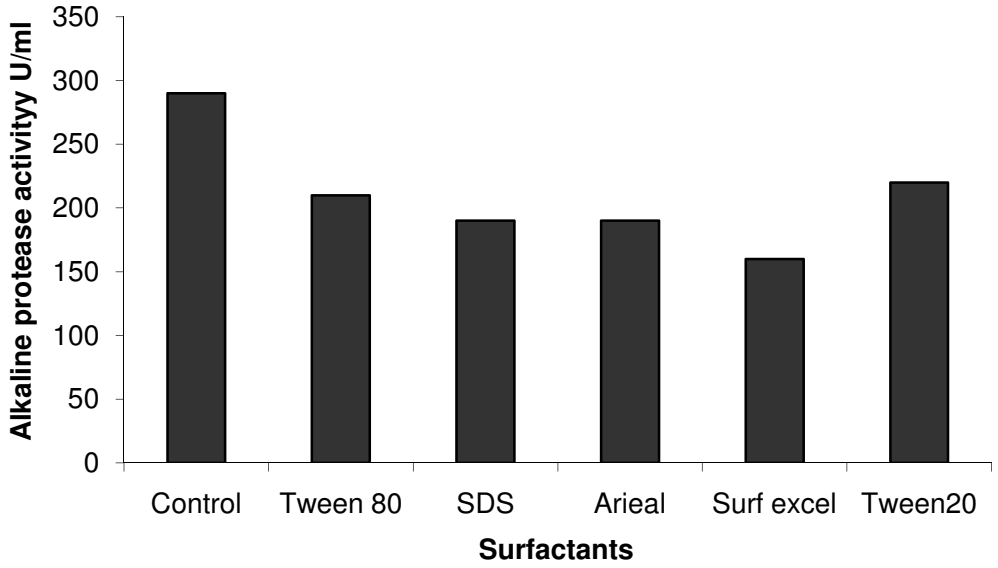

Fig 14 Effect of surfactants on alkaline protease production by mutant *Bacillus licheniformis* (Bl8)

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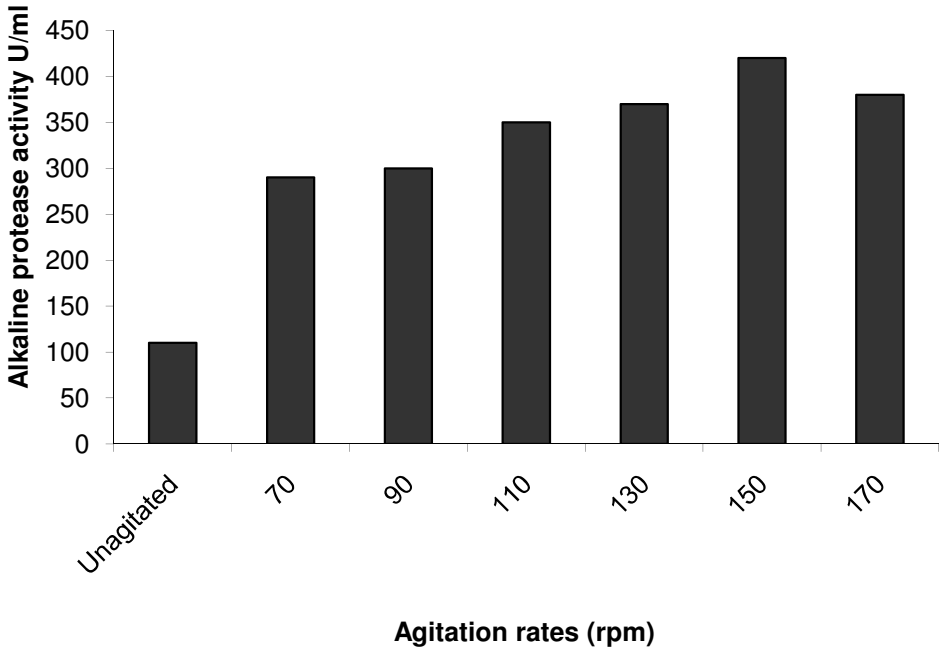

Fig 15 Effect of agitation rates on alkaline protease production by mutant *Bacillsus licheniformis* **(Bl8)**

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