Biological Sciences

Pak. J. Sci. Ind. Res. 2008 **51**(3) 150-157

Stimulatory Effect of Medium Ingredients on Alkaline Protease Production by *Bacillus licheniformis* N-2 and Compatibility Studies With Commercial Detergents

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(received April 11, 2008; revised June 3, 2008; accepted June 12, 2008)

Abstract. Suitable concentration of ingredients of the growth medium played a vital role in production of alkaline protease by *Bacillus licheniformis*. Maximum enzyme activity (875.05 PU/ml) was achieved when the bacterium was grown in the medium containing glucose (1%), soybean meal (1%), K_2HPO_4 (0.5%), $MgSO_4.7H_2O$ (0.05%), NaCl (0.05%), $CaCl_2.2H_2O$ (0.05%) at 37 °C on 24 h incubation period with agitation of 140 rpm in shake flask cultures. More than 1% glucose decreased the enzyme production. The protease had excellent stability with wide range of commercial detergents such as Ariel, Bonus, Bright Total, Surf Excel, Wheel and non-branded detergents, recommending its use as an effective additive in detergent formulation.

Keywords: medium ingredients, detergent compatibility, *B. licheniformis* N-2, alkaline protease

Introduction

Proteases are one of the most important groups of industrial enzymes used in pharmaceutical industry and in food industry for peptide synthesis, in leather industry for de-hairing and in detergent industry as an additive of detergent formulation (Joo and Chang, 2005; Pastor *et al.,* 2001). Alkaline proteases are known to constitute 60-65% of the global industrial market among various types of proteases (Banerjee *et al.,* 1999). Alkaline proteases are produced by a wide range of microorganisms including bacteria, mould and yeast. Currently, a large portion of commercially available proteases is derived from *Bacillus* strains because of their high pH and temperature stability (Gupta *et al.,* 2002; Joo *et al.,* 2002).

The fermentation medium form the environment in which the microorganisms live, reproduce and carry out their specific metabolic reactions to produce useful products. Two distinct biological requirements are considered in most of the industrial fermentation processes for medium design, where the product is something other than the cell mass itself. First, the nutrient has to be supplied to establish the growth of the microorganism. Second, proper nutritional conditions have to be provided to maximize the product formation. It is also well established that extracellular protease production in microorganisms is greatly influenced by media components, especially carbon and nitrogen sources, metal ions and physical factors such as pH, temperature, dissolved oxygen and incubation time (Nadeem, *et al*. 2006; Oberoi *et al.,* 2001; Kuar *et al.,* 2001; Razak, *et al.* 1994; Moon and Parulekar, 1993). The cost of the growth medium is another significant parameter for making the production process industrially viable. Approximately 30-40% of the production cost of the industrial enzyme is estimated to be accounted for by the cost of the growth medium (Gessesse, 1997). Therefore, selection of the right medium ingredients and their concentrations optimization have become the need of today for high yield of desirable enzymes by fermentation.

Considering these facts, the effects of different concentrations of carbon and nitrogen sources as well as metal ions concentrations were studied to maximize the yield of alkaline protease by locally isolated *Bacillus licheniformis* N-2. Compatibility studies of alkaline protease with different detergents were also conducted to observe its commercial exploitation as an additive in detergent formulation for laundry industry.

Materials and Methods

Microorganism and culture maintenance. A proteolytic strain identified as *Bacillus licheniformis* N-2 was isolated from decaying organic soil sample (Nadeem *et al*., 2007) and the culture was grown on nutrient agar slants at 37 °C for 24 h and preserved at 4 °C for one month. The preserved culture was revived on fresh nutrient agar slants after every one month for subsequent experiments.

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***Inoculum preparation*.** The inoculum was prepared by transferring a loopful of 24 h old culture of *B. licheniformis* N-2 into 50 ml of inoculum medium consisting of glucose (0.5%), soybean meal (1%), K_2HPO_4 (0.3%), $MgSO_4.7H_2O$ (0.05%), NaCl (0.05%), and $CaCl_2.2H_2O$ (0.05%). The inoculated medium was incubated in water bath shaker (Eyela, Japan) for 24 h at 37 °C temperature and 140 rpm agitation speed for the propagation of bacteria up to 10^{8-10} cells/ml.

Fermentation process. The initial growth medium composed of glucose (1%), soybean meal (1%), K_2HPO_4 (0.3%), $MgSO_4.7H_2O$ (0.05%), NaCl (0.05%) and $CaCl_2.2H_2O$ (0.05%). It inoculated with 1% (v/v) of 24 h old inoculum broth. The pH of the medium was adjusted at 10 with 1N HCl/NaOH before sterilization at 121°C for 15 min. The inoculated medium was incubated in water bath shaker (Eyela, Japan) for 24 h at 37 °C and 140 rpm. Thereafter, the fermented broth was centrifuged at 9000 x g for 10 min at 4 °C to get clear supernatant enzyme solution.

Optimization of concentrations of medium ingredients. Different concentration levels of carbon and nitrogen sources as well as inorganic elements were optimized to make the process cost effective for alkaline protease production by *B. licheniformis* N-2.

***Effect of glucose concentrations*.** Effect of various glucose concentrations (0.2, 0.4, 0.6, 0.8, 1.0, 1.25 and 1.50%) were studied to find the suitable glucose concentration for the maximum yield of alkaline protease by *B. licheniformis* N-2.

***Effect of defatted soybean meal concentrations*.** Effect of different concentrations of defatted soy bean meal viz. 0.25, 0.50, 0.75, 1.0, 1.25, 1.5, 1.75 and 2.0% on alkaline protease production was determined by incubating the growth medium at 37 °C for 24 h in water bath shaker (Eyela, Japan) with agitation speed of 140 rpm.

***Effect of metal ions on alkaline protease production*.** Effects of various metal ions in the form of K_2HPO_4, $MgSO_4.7H_2O$, NaCl, and $CaCl_2.2H_2O$ on alkaline protease production were studied to find the suitable concentration level of each element in the growth medium. The concentration of each element was used in the range of 0.01% to 0.07% (w/v) except K_2HPO_4, with concentration varying from 0.1% to 0.7%. All the experiments were performed in triplicate by changing concentration of one element but keeping the concentration of others constant.

Analytical procedures: *Total protein contents*. Total protein contents of samples were determined by the method of Lowry *et al*. (1951) using bovine serum albumin (BSA) as the reference standard.

***Total biomass determination*.** The cell biomass was determined from a known amount of sample centrifuged at 9000 x g for 10 min at 4 °C. The cell biomass pellet was washed with sterilized normal saline three times to remove the suspended particles. The washed cell pellet was dried at 105 °C till the constant weight is obtained.

***Determination of alkaline protease activity*.** Proteases activity was determined by a slightly modified method of Yang and Huang (1994). The reaction mixture containing 1 ml of 1.0% casein solution in 0.05 M glycine-NaOH buffer having pH 11 and 1 ml of enzyme solution were incubated at 60 °C for15 minutes and the reaction was then stopped with 3 ml of 10% tri-chloroacetic acid (TCA). After 10 min the entire mixture was centrifuged at 9000 x g for 10 min at 4.0 °C and absorbance of the liberated tyrosine was measured with respect to sample blank at 280 nm. One proteolytic unit (PU) was defined as the amount of the enzyme that released 1µg of tyrosine under the assay conditions.

Compatibility of alkaline protease with various commercial detergents. The compatibility of *B. licheniformis* N-2 protease with different detergents was studied using Ariel, Bonus, Bright Total, Surf Excel, Wheel and non-branded detergent. All the detergents were diluted with distilled water to give final concentration of 7 mg/ml to stimulate washing conditions. The enzyme in the detergents were deactivated by heating the detergents solution at 100 °C for 10 min. After that, a known quantity of alkaline protease in the presence of 5 mM and 10 mM $CaCl_2$ was added to each detergent solution separately. The enzyme mixtures were incubated at 40 °C for different time intervals (15-120 min) and then the residual activity was determined according to the standard assay procedure. The enzyme activity was taken as 100% of the enzyme mixture without incubation.

Chemical and statistical analysis. All the chemicals used in this study were of analytical grade. Each experiment was conducted in triplicate and the standard deviation (SD) was calculated by using Microsoft Excel Program.

Results and Discussion

Effect of carbon concentrations on alkaline protease production. As glucose was found to be the best carbon source (un-published data) for alkaline protease production by *B. licheniformis* N-2, therefore, different concentrations of glucose varying from 0.2% to 1.5% over the control (without external source of glucose) were used to determine the optimum concentration for the maximum yield of alkaline protease. The highest protease production (678.21 PU/ml) and cell biomass (3.71 g/l) was observed at 1.0% concentration

(w/v) of glucose (Table 1). A decrease in enzyme production was observed at lower or higher concentrations other than the optimum. The results indicated that proper concentration level of glucose plays significant role in enhancing the production of alkaline protease and growth of the *B. licheniformis* N-2. Higher concentrations repressed the growth and enzyme production might be due to the catabolic repression, or substrate inhibition, a traditional property of the batch fermentation processs. A decrease in enzyme production was also observed at higher concentration of glucose (> 8 k/g m^3) by Calik *et al*. (2003). Adinarayana *et al.* (2003) found maximum enzyme yield at 0.5% glucose concentration by *Bacillus subtilis*. On the other hand, He *et al.* (2003) reported highest protease activity (2508U/ml) by *Bacillus* sp. EL31410 at 4 % glucose concentration level. The difference in glucose concentration among different *Bacillus* species might be due to the difference in physiological and metabolic characteristics. It is well known that there is big difference among the metabolic characteristics of the same species isolated from different sources.

Effect of soybean meal concentration on alkaline protease production. Among different nitrogen sources tested for alkaline protease production by *B. licheniformis* N-2 (data not shown), soybean meal exhibited a prominent effect on protease production and growth of the *bacterium*. Soybean meal had stimulatory effect on alkaline protease production and maximum yield (680.02 PU/ml) was obtained at 1.0% (w/v) concentration (Table 2). Further increase in concentration considerably repressed the growth and protease production. A similar finding was observed by Elibol and Moreira (2005) who observed maximum enzyme production at 1.0% concentration level by a marine bacterium *Teredinobacter turnirae*. Optimum alkaline protease yield was reported at 1.5% concentration of soybean meal in the growth medium

Table 1. Effect of glucose on alkaline protease production by *B. licheniformis* N-2 after 24 h incubation at 37 °C and 140 rpm

Glucose conc. (%)	Cell biomass (g/l)	Total protein (mg/ml)	Final pH of the medium	Enzyme activity (PU/ml)
Control	2.68±0.03	2.25±0.02	8.61±0.12	315.22±7.22
0.20	2.79±0.02	2.27±0.06	8.56±0.11	375.35±6.38
0.40	3.15±0.04	2.43±0.04	8.51±0.13	405.81±9.11
0.60	3.41±0.07	2.85±0.11	8.43±0.08	500.06±5.78
0.80	3.58±0.05	3.09±0.05	8.25±0.10	586.19±6.04
1.00	3.71±0.06	3.34±0.12	8.18±0.12	678.21±7.12
1.25	3.62±0.07	3.12±0.10	8.17±0.11	635.36±7.89
1.50	3.31±0.08	2.74±0.07	8.21±0.09	445.74±8.06

control: without external source of glucose; each value is an average of three parallel replicates ±indicates standard deviation among the replicates

Table 2. Effect of defatted soybean meal on alkaline protease production by *B. licheniformis* N-2

Soybean meal conc. (%)	Cell biomass (g/l)	Total protein (mg/ml)	Final pH of the medium	Enzyme activity (PU/ml)
Control	2.82±0.13	2.02±0.05	8.13±0.07	255.35±5.54
0.25	2.95±0.09	2.45±0.03	8.16±0.10	315.33±8.14
0.50	3.17±0.10	2.88±0.08	8.19±0.07	425.08±4.03
0.75	3.35±0.07	3.14±0.11	8.18±0.12	534.32±5.42
1.00	3.74±0.11	3.35±0.10	8.19±0.11	680.02±9.07
1.25	3.73±0.08	3.45±0.06	8.21±0.06	666.11±4.44
1.50	3.73±0.10	3.52±0.05	8.20±0.08	635.08±5.06
1.75	3.65±0.07	3.47±0.04	8.19±0.05	612.46±9.11
2.00	3.52±0.12	3.44±0.07	8.21±0.10	561.43±10.35

control: without external source of defatted soybean meal; each value is an average of three parallel replicates ± indicates standard deviation among the replicates

from *Bacillus* sp. I-312 and *Bacillus horikoshii* (Joo and Chang, 2005; Joo *et al.*, 2002). In their studies on protease production, Laxman *et al*. (2005) observed maximum yield at 2% soybean meal concentration from *Conidiobolus coronatus* while Sutar *et al.* (1992) reported highest protease activity in the medium containing 4% soybean meal. All these findings indicated that different concentration levels of soybean meal influenced the metabolic processes of each microorganism involved in fermentation process. Therefore, capacity of utilization of soybean meal varies from species to species or even in the same species isolated from different environments.

Effect of different metal ions on alkaline protease production: *Effect of K_2HPO_4 concentrations on alkaline protease production*. Effects of various metal ions on cell growth and enzyme production were studied stepwise for the optimum yield of alkaline protease by *B. licheniformis* N-2. For this purpose, the basal medium was amended with different concentrations of K_2HPO_4 varying from 0.1% to 0.7% against control (without external source of K_2HPO_4). Maximum alkaline protease yield (873.25 PU/ml) was observed at 0.5% initial concentration (w/v) of K_2HPO_4 that gave 2.1 fold increases in enzyme production over the control (Table 3). Theses results are in agreement with the earlier findings which showed enhancement of protease activity in the presence of metal ions (Adinarayana *et al.*, 2003; Thangam and Rajkumar, 2002). Our findings are also supported by Calik *et al.* (2003) who found 1.55 fold higher enzyme activity in the presence of phosphate ions by *Bacillus subtilis.* Rahman *et al*. (2005) observed 39% increases in enzyme production in the presence of 2mM K^{1+} ions in the growth medium by *Pseudomonas aeruginosa*. All these findings indicated that potassium and phosphate ions provided by K_2HPO_4 play an important role in growth of microbes and enzyme production because these metal ions are the major constituent of nucleotide, nucleic acid and phospholipids.

Effect of $MgSO_4.7H_2O$ concentration on alkaline protease production. Magnesium is an essential cofactor for many of the glycolytic enzymes and depletion of magnesium in the culture broth inhibits glycolysis (Dombek and Ingram, 1986). Therefore, various concentrations of $MgSO_4.7H_2O$ ranging from 0.01% to 0.07% were studied to observe the suitable concentration of Mg^{2+} ions for maximum growth and alkaline protease production by *B. licheniformis* N-2. The results revealed 1.1 fold increase in enzyme production (873.22 PU/ml) in the presence of 0.05% $MgSO_4.7H_2O$ concentration in the growth medium (Table 4). The results are supported by Jasvir *et al.* (1999) who observed increase in enzyme production in the presence of Mg^{2+} ions. Rahman *et al.* (2005) also observed 14% increase in enzyme production in the presence of 2mM Mg^{2+} ions. However, a decrease in alkaline protease production was observed by Calik *et al*. (2003) in the presence Mg^{2+} ion that might be attributed to the difference in medium composition as well as in the microorganism.

Effect of NaCl concentration on alkaline protease production. The effect of Na^{+} ions on alkaline protease production and growth of *B. licheniformis* N-2 was studied by amending the cultivation medium with different concentrations (w/v) of NaCl ranging from 0.01% to 0.07%. Maximum enzyme activity (874.61 PU/ml) was obtained at 0.05% concentrations of NaCl which indicated 1.16 fold increase in the enzyme yield as compared to the control (Table 5). Thereafter, a decrease in alkaline protease yield was observed with further increase in concentration of NaCl. Shanmughapriya *et al.* (2007) obtained highest protease activity at 3 % NaCl

Table 3. Effect of K_2HPO_4 on alkaline protease production by *B. licheniformis* N-2

K_2HPO_4 conc. (%)	Cell biomass (g/l)	Total protein (mg/ml)	Final pH of the medium	Enzyme activity (PU/ml)
Control	3.11±0.04	2.93±0.08	7.15±0.11	415.19±8.85
0.10	3.15±0.12	2.96±0.04	7.48±0.08	470.21±9.25
0.20	3.37±0.07	3.25±0.06	8.06±0.10	551.83±6.66
0.30	3.72±0.15	3.36±0.07	8.21±0.06	678.93±8.12
0.40	3.81±0.10	3.42±0.03	8.33±0.09	725.43±11.05
0.50	3.84±0.08	3.45±0.06	8.54±0.04	873.24±12.67
0.60	3.82±0.06	3.47±0.05	8.67±0.06	678.36±8.98
0.70	3.78±0.12	3.46±0.05	8.81±0.08	619.54±10.08

control: without external source of K_2HPO_4; each value is an average of three parallel replicates ± indicates standard deviation among the replicates

concentration by a marine isolated *Roseobacter* sp. This big variation with our findings might be due to difference in the nature of the species.

Effect of $CaCl_2.2H_2O$ concentration on alkaline protease production. Effect of Ca^{2+} ions on protease production and growth of *B. licheniformis* N-2 were investigated by using $CaCl_2.2H_2O$ as a Ca^{2+} ion source of varying concentration from 0.01 to 0.07% over control (without external source of $CaCl_2.2H_2O$). The results revealed that 0.05% concentration affected the growth and protease production noticeably and enhanced 1.3 fold enzyme yield over the control (Table 6). Ca^{2+} ions were studied as an effective inducer for enzyme production by Shafee *et al.* (2005). Vidyasagar *et al.* (2006) investigated the similar findings and observed maximum enzyme production at 200mM Ca^{2+} ions by *Halogeometricum* sp. TSS101. Our results are also in good agreement with Mabrouk *et al.* (1999) who observed 26.6% increase in enzyme production in the presence of 0.07% $CaCl_2.2H_2O$ over the control in the growth medium. All these finding indicated that Ca^{2+} ions had stimulatory effect on protease production and the presence of Ca^{2+} ion in the fermented broth might also stabilize the structure of extracellular alkaline protease.

Compatibility studies with various commercial detergents. The compatibility of alkaline protease with different commercial detergents (brand names: Arial, Bonus, Bright Total, Wheel and un-branded detergent) were studied in the absence and presence of different concentrations (5 mM and 10 mM) of $CaCl_2.2H_2O$. Alkaline protease from *B. licheniformis* N2 showed a wide range of compatibility with different detergents. Maximum compatibility was observed with Bright Total and Wheel up to 58% and 58.06% after 120 min of incuba-

Table 4. Effect of $MgSO_4.7H_2O$ on alkaline protease production by *B. licheniformis* N-2 after 24 h incubation at 37 °C and 140 rpm

$MgSO_4.7H_2O$ conc. (%)	Cell biomass (g/l)	Total protein (mg/ml)	Final pH of the medium	Enzyme activity (PU/ml)
Control	3.47±0.08	3.37±0.03	8.53±0.08	795.12±10.02
0.01	3.54±0.10	3.38±0.05	8.52±0.11	815.46±12.41
0.02	3.64±0.12	3.38±0.05	8.54±0.07	829.12±11.67
0.03	3.72±0.11	3.40±0.04	8.54±0.12	842.37±15.08
0.04	3.78±0.07	3.44±0.08	8.53±0.06	861.19±8.92
0.05	3.85±0.10	3.46±0.05	8.55±0.10	873.22±9.44
0.06	3.84±0.06	3.48±0.04	8.56±0.06	862.03±10.11
0.07	3.83±0.07	3.48±0.06	8.55±0.05	846.27±9.18

control: without external source of $MgSO_4.7H_2O$; each value is an average of three parallel replicates ± indicates standard deviation among the replicates

Table 5. Effect of NaCl on alkaline protease production by *B. licheniformis* N-2 after 24 h incubation at 37 °C and 140 rpm

NaCl conc. (%)	Cell biomass (g/l)	Total protein (mg/ml)	Final pH of the medium	Enzyme activity (PU/ml)
Control	3.42±0.12	3.38±0.04	8.51±0.03	755.02±8.88
0.01	3.52±0.11	3.43±0.08	8.51±0.07	801.22±15.32
0.02	3.57±0.08	3.42±0.03	8.53±0.10	815.08±16.24
0.03	3.68±0.10	3.44±0.05	8.48±0.11	838.18±12.58
0.04	3.81±0.11	3.44±0.07	8.53±0.05	855.81±15.49
0.05	3.84±0.12	3.45±0.05	8.54±0.08	874.61±11.72
0.06	3.84±0.09	3.48±0.06	8.59±0.07	855.35±14.28
0.07	3.80±0.08	3.50±0.08	8.43±0.09	842.19±9.33

control: without external source of NaCl; each value is an average of three parallel replicates ± indicates standard deviation among the replicates

Table 6. Effect of $CaCl_2.2H_2O$ on alkaline protease production by *B. licheniformis* N-2 after 24 h incubation at 37 °C and 140 rpm

$CaCl_2$. $2H_2O$ conc. (%)	Cell biomass (g/l) (gl/l)	Total protein (mg/ml)	Final pH of the medium`	Enzyme activity (PU/ml)
Control	3.02±0.06	3.23±0.11	8.47±0.06	675.22±10.20
0.01	3.18±0.12	3.35±0.12	8.48±0.08	701.28±8.88
0.02	3.36±0.07	3.42±0.13	8.53±0.02	726.17±12.05
0.03	3.62±0.06	3.44±0.08	8.54±0.05	755.71±10.72
0.04	3.78±0.08	3.44±0.10	8.52±0.07	806.45±7.83
0.05	3.84±0.10	3.45±0.09	8.56±0.03	875.05±10.10
0.06	3.91±0.07	3.53±0.05	8.61±0.05	870.12±11.23
0.07	3.88±0.08	3.55±0.06	8.62±0.04	835.43±12.36

control: without external source of $CaCl_2$. $2H_2O$; each value is an average of three parallel replicates ± indicates standard deviation among the replicates

tion at 40 °C without Ca^{2+} ions (Fig. 1). However, least compatibility was determined against Arial up to 7% in the absence of Ca^{2+} ions after 120 min incubation. These findings indicate that addition of suitable stabilizers are essential for proper utilization of alkaline protease against commercial detergents. The enzyme retained more than 50% its stability with most of the detergents in the presence of 5mM of Ca^{2+} ions whereas the maximum stability was determined with Bright Total (72%) followed by Wheel (68.11%) as shown in Fig. 2. After supplementation with 10 mM of Ca^{2+} ions, the enzyme retained more than 65% residual activity against all the detergents even after 120 min incubation at 40 °C (Fig. 3). However, maximum stability about 93.11% was found against Wheel followed by Bright Total (88.34%). Adinarayana *et al.* (2003) reported that alkaline protease retained 65% activity in the presence of Wheel after 3 h of incubation at 60 °C followed by Nirma (58%), Surf Excel (56%) and Ariel (52%). Bhosale *et al*. (1995) reported that protease production by *Conidiobolus coronatus* retained 16 % activity in Revel, 11.4% activity in Ariel and 6.6% activity in Wheel in the presence of 25 mM $CaCl_2.2H_2O$ at 50 °C. Nascimento and Martins (2006)

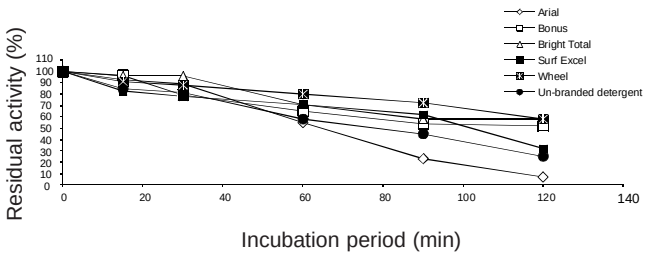

Fig. 1. Compatibility of alkaline ptotease activity from *B. licheniformis* N-2 with different commercial solid detergents. Enzyme activity of control samples (without incubation) was taken as 100%.

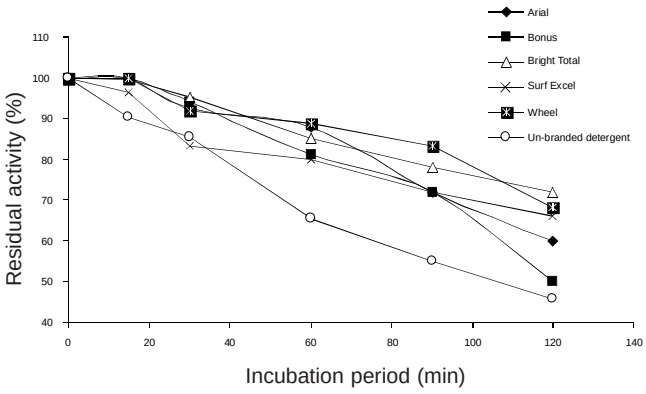

Fig. 2. Compatibility of alkaline protease activity from *B. licheniformis* N-2 with different commercial detergents in the presence of 5 mM $CaCl_2$. $2H_2O$. Enzyme activity of control samples (without incubation) was taken as 100%.

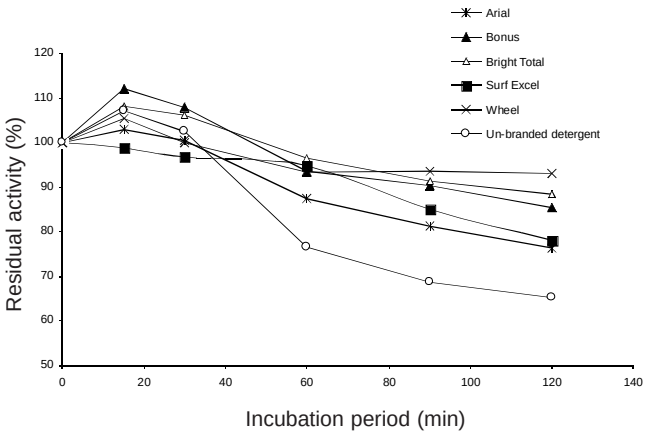

Fig. 3. Compatibility of alkaline protease activity from *B. licheniformis* N-2 with different commercial detergents in the presence of 10 mM $CaCl_2$. $2H_2O$. Enzyme activity of control samples (without incubation) was taken as 100%.

found more than 85% activity in the presence of Ca^{2+} ions after 1 h incubation at 60 °C. Comparing these results, the alkaline protease produced by *B. licheniformis* N-2 was found to be significantly more stable over a broad range of commercial detergents.

Conclusion

Alkaline protease production and growth of *B. licheniformis* N-2 under submerged fermentation were found to be influenced by different concentrations of glucose, soybean meal and various metal ions in the growth medium. The maximum protease production (875.05 PU/ml) was achieved by employing optimized concentrations of glucose (1%), soybean meal (1%), K_2HPO_4 (0.5%), $MgSO_4.7H_2O$ (0.05 %), NaCl (0.05%), $CaCl_2.2H_2O$ (0.05%) after 24 h incubation at 140 rpm. The compatibility studies of alkaline protease with different commercial detergents in the presence and absence of Ca^{2+} ions indicated that the *B. licheniformis* N-2 protease is suitable for detergent formulation. More than 65 % stability was observed with various commercial detergents in the presence of 10 mM $CaCl_2.2H_2O$. Collectively, all these results may justify the possibilities of production of alkaline protease by *B. licheniformis* N-2 for its commercial exploitation in detergent industry.

Acknowledgement

The authors are thankful to the Ministry of Science and Technology (MoST), Government of Pakistan for its financial support, under the PSDP project allocation No.31, to carry out this research work.

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