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Full Length Research Paper

The influence of carbon and nitrogen supplementation on alpha amylase productivity of *Bacillus amyloliquefaciens* IIB-14 using fuzzy-logic and two-factorial designs

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This study is concerned with the production and optimization of alpha amylase by *Bacillus amyloliquefaciens* IIB-14 in the presence of additional carbon and nitrogen sources using solid state fermentation. Alpha amylase is of special importance in the starch fermentation industry, as it has significant commercial applications in starch processing, beverages, foods, bread and baking, medicines, textile, paper, pharmaceuticals, sugar and detergent industries. Maltose, glucose, lactose and soluble starch were supplemented as carbon sources. Urea, peptone, yeast extract and nutrient broth as organic nitrogen sources while NH_4NO_3, $(NH_4)_2SO_4$, $NaNO_3$ and KNO_3 as inorganic nitrogen sources were evaluated. Among them, a marked improvement in enzyme production (70.15 U/mg/min) was obtained with lactose, yeast extract and NH_4NO_3 at a level of 1.5, 1 and 1%, respectively. The performance of fuzzy-logic system control was found to be highly promising for the improved substrate conversion rate of more than 75%. The process parameters were further identified using Plackett-Burman design. The correlation (1.045*E*+0025) among variables demonstrated the model terms as highly significant (*HS*) indicating commercial utility of the culture used ($p<0.05$). The maximal enzyme activity (84.24 U/mg/min) of crude enzyme was found at pH 7 and temperature 70°C incubated for 30 min.

Key words: Alpha amylase, *Bacillus amyloliquefaciens*, solid state culture, carbon and nitrogen supplementation, fuzzy-logic control, two-factorial experimental design.

INTRODUCTION

Amylases (α-amylase, β-amylase and glucoamylase) are one of the most important families of enzymes in the field of biotechnology (Arikan, 2007). They can be divided into two categories, endoamylases and exoamylases. Endoamylases such as alpha amylase cleaves α-1, 4 glycosidic bonds in a random manner present in the inner part (endo) of amylose or amylopectin chain of the starch molecule. Exoamylases either cleave α-1, 4 glycosidic bonds such as β-amylase or cleave both α-1, 4 and α-1, 6 glycosidic bonds like amyloglucosidase or glucoamylase and α-glucosidase from the non-reducing end of the starch molecule (Agrawal et al., 2005). Alpha-amylase (endo-1, 4-α-D-glucan glucanohydrolase, *EC 3.2.1.1*) is an extra-cellular endo-enzyme that randomly cleaves α-1, 4 glucosidic bonding of linear amylose and

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branching amylopectin chains of the starch molecule (Kiran and Chandra, 2008; Tanyildizi and Dursun, 2011; Rameshkumar and Sivasudha, 2011). Alpha amylase can be produced by different species of microorganisms. However, bacterial and fungal alpha amylases have dominated applications in many industrial processes. Bacterial alpha amylases are limited to the genus *Bacillus* (Gangadharan et al., 2011). Among them, *B. subtilis, B. licheniformis, B. amyloliquefaciens and B. stearothermophilus* were reported to be more prominent for the production of alpha amylase (Aiyer, 2005; Akcan et al., 2011). Of the starch hydrolyzing enzymes, the alpha amylase is of special importance as it is responsible for the solubilization of starch (Sudha, 2012; Sharanappa et al., 2011). This enzyme has significant commercial applications in starch processing, food, bread and baking, textile, paper, pharmaceuticals, sugar and detergent industries (Asgher et al., 2007; Demirkan, 2011; Li et al., 2011). Both solid state (SSF) and submerged fermentations (SmF) could be used for the production of alpha amylase. However, SSF has numerous advantages over SmF, including superior productivity, simple technique, low capital investment, low energy requirement and less waste water output, better product recovery and lack of foam build- up (Babu and Satyanarayana, 1995).

In SSF, the solid substrate not only supplies the nutrients to the culture, but also serves as an anchorage for the microbial cells (Baysal et al., 2003). The optimizations of chemical and physical parameters have inductive role on the production of alpha amylase (Akcan, 2011). The application in the starch industry does not require high-purity amylases and generally makes use of crude or partially purified enzyme preparations (Asgher et al., 2007). However, it is significant to obtain enzymes with higher specific activity for their kinetic characterization (Sodhi et al., 2005). The optimal temperature for maximum enzyme activity is from 60 to 90°C. The bacterial cultures normally grow at pH from 4.5-9.5 while enzyme activity remains optimal at pH 5.5 to 6 (Rao and Satyanarayana, 2003). The metabolites are usually analyzed during or after the completion of a process.

The secretion of amylolytic enzyme is generally characterized by a flexible, unsteady, limited and non-linear dynamic operation (Takasaki et al., 1994). The operating variables must not be altered to obtain a consistent product quality. However, changes in product yield may arise from deviation in specified transfer routes or variation in the impurities of medium. The inherent non-linearity of alpha amylase productivity renders its control difficult (Declerck et al., 2003; Lulko et al., 2007). So there is a need to fine-tune the process by intelligent control. Fuzzy logic is an approach to control the process performance and thus, has become a popular handling tool in fermentation-based technology. The present work is concerned with the production and optimization of alpha amylase by *Bacillus amyloliquefaciens* IIB-14 in the presence of additional carbon and nitrogen sources under solid state fermentation using a fuzzy-logic control system. A two factorial experimental design i.e., Plackett-Burman method was used to identify significant variables influencing hyper enzyme secretion in a batch process.

MATERIALS AND METHODS

Organism

B. amyloliquefaciens IIB-14 was used in this study. The strain was obtained from the available stock culture of the Institute of Industrial Biotechnology (IIB), GC University Lahore. The culture was maintained on the nutrient broth agar (pH 7) slants and stored at 4°C.

Preparation of inoculum

Fifty milliliter of inoculum medium (pH 7) containing nutrient broth 0.8% (w/v) was transferred to a 250 ml Erlenmeyer flask. The flask was cotton plugged and autoclaved at 121°C (15 psi) for 15 min. After cooling to room temperature, a loopful of bacteria was aseptically transferred to each flask. The flasks were kept in a rotary shaking incubator at 40°C and 200 rpm for 24 h.

Process parameters and fermentation technique

Alpha amylase fermentation was carried out under solid state fermentation with wheat bran. Ten grams of wheat bran was weighed into 250 ml Erlenmeyer flasks and 12.5 ml of 0.02 M phosphate buffer (pH 7.2) was added, cotton plugged and autoclaved at 121°C (15 psi) for 15 min. The flasks were cooled to room temperature and then inoculated with 1.0 ml of 24 h grown bacterial culture (2.35×10^7 CFU) under sterile conditions and incubated at 40°C under static condition for 72 h. The contents of flasks were shaken twice a day to ensure maximal oxygen supply and a better bacterial growth. All the experiments were run parallel in triplicates.

Enzyme extraction

After the incubation period, 100 ml of 0.02 M phosphate buffer (pH 7.2) was added to each flask. Contents were mixed thoroughly by shaking for 1 h at 40°C in a rotary shaker at 200 rpm. At the end of the extraction, the suspension was centrifuged at 6000 rpm for 15 min; the supernatant was collected and used as the crude enzyme for further analysis.

Assay of alpha amylase

Alpha amylase activity was determined as described by Rick and Stegbauer in 1974. The reaction mixture containing 0.5 ml of 1% (w/v) soluble starch and 0.5 ml of crude enzyme solution was incubated for 10 min at 60°C. A blank containing 0.5 ml of 1% (w/v) soluble starch and 0.5 ml of distilled water was incubated in parallel. The reaction was stopped by adding 1 ml of 3, 5-dinitrosalicylic acid (DNS) solution to each tube followed by heating in a boiling water bath for 5 min and cooling at room temperature and then 8 ml of distilled water was added to each tube. The absorbance of each solution was measured at 546 nm using a

spectrophotometer and converted to milligram of maltose using the standard maltose curve. One enzyme unit is defined as the amount of enzyme that hydrolyses 1 mg of starch (1 %) in 10 min at 60°C.

Determination of proteins

Total protein content was estimated by taking 0.1 ml of the enzyme extract and 5 ml of Bradford reagent in a test tube according to method of Bradford 1976. A blank containing 0.1 ml of distilled water and 5 ml of the Bradford reagent was run in parallel. The absorbance was measured at 595 nm using spectrophotometer. Protein content was estimated using standard BSA (Bovine Serum Albumin) curve.

Effect of carbon and nitrogen supplementation on alpha amylase production

Effect of different carbon sources (glucose, maltose, lactose and soluble starch) and organic and inorganic nitrogen sources were evaluated for the production and optimization of alpha amylase using solid state fermentation. Organic nitrogen sources include yeast extract, nutrient broth, urea and peptone, while inorganic nitrogen sources include NH_4NO_3, $(NH_4)_2SO_4$, $NaNO_3$ and KNO_3.

Operating fuzzy-logic control system

The step-wise fermentation process was fine-tuned and operated using a fuzzy logic control system. The input variables were error and error rate for enzyme production (*P*). The lower and higher values of operating parameters (*Q*) were selected as output variables. The discourse comprised of three sets that is, negative (*NE*), positive (*PO*) and zero (*ZE*). The centroid method was used to obtain a lower or higher *P*-value. An initial rule base was made by stating a number of rules that were operating in the reaction vessel. The rules were written and checked for their correctness by an interactive process to exert control action at lower *Q* values.

Optimal conditions for alpha amylase activity

The optimal activity of the alpha amylase was determined as a function of pH and temperature (Asgher et al., 2007). The effect of different pH (5, 5.5, 6, 6.5, 7, 7.5) and temperature (50, 55, 60, 65, 70, 75°C) at different time periods (5, 10, 15, 20, 25, 30, 35 min) was investigated.

Application of Plackett-Burman two-factorial design

Duncan's multiple range tests (Spss-18, version 6.8) were applied under one-way ANOVA and the treatment effects were compared (Snedecor and Cochran, 1980). Significant variables were identified using a two factorial experimental design (Plackett and Burman, 1946). Variables were denoted at two widely spaced intervals and the effect of individual parameters on enhanced alpha amylase productivity was calculated by the following equations:

$$E_o = (\Sigma M_+ - \Sigma M_-) / N \quad (1)$$

$$E = \beta_1 + \Sigma \beta_2 + \Sigma \beta_3 + \beta_{123} \quad (2)$$

In Equation 1, E_o is the effect of first parameter under study while M_+ and M_- are responses of enzyme secretion. N is the total number of optimizations. In Equation 2, E is the significant parameter, β_1 is the linear coefficient, β_2 the quadratic coefficient and β_3 is the interaction coefficient.

RESULTS AND DISCUSSION

The influence of carbon and nitrogen supplementation on alpha amylase productivity of *Bacillus amyloliquefaciens* IIB-14 was investigated. The cell growth and production of alpha amylase by *Bacillus* species is potentially affected by the nature and concentration of carbon source (Welker and Campbell, 1967). Figure 1a highlights the effect of different carbon sources such as maltose, glucose, lactose and soluble starch on the production of alpha amylase. These sugars were added to the fermentation medium at the level of 1%. Extremely low production of enzyme was obtained with glucose (35.12 U/mg/min), probably due to the fact that easily metabolizable carbon source promoted the growth of the microorganism with significant reduction of enzyme production. However, lactose gave maximum production of alpha amylase (57.92 U/mg/min) and protein content (539 µg/ml) due to the fact that lactose was a good stimulator of alpha amylase and was slowly metabolized with significant accumulation of inducible enzyme in the fermentation medium (Kelly et al., 1997). Therefore, lactose was found to be optimal for further studies. Lactose concentrations were varied from 0.5 to 3% as shown in Figure 1b. Minimum production of enzyme (53.43 U/mg/min) was obtained when 0.5% lactose was used. However, it was gradually increased with increase in the concentration of lactose from 0.5 to 1% and sharply increased with 1.5% lactose. The lactose at the level of 1.5% gave optimal production of alpha amylase (61.93 U/mg/min) and protein content (545 µg/ml). By further increasing the level of lactose (2%), there was a marked decline in the production of alpha amylase (55.62 U/mg/min). It was further decreased to 46.12 U/mg/min at concentration of 3%. This behavior is attributed to the fact that at high concentration of carbon source, its consumption is rapid, resulting in the release of metabolic toxic wastes, which inhibit the growth of bacteria and hence the production of alpha amylase. Thus, the addition of 1.5% level of lactose was found to be optimal for the production of alpha amylase.

Nitrogen sources greatly affect the production of alpha amylase (Birol et al., 1997). Different organic nitrogen sources such as urea, peptone, yeast extract and nutrient broth were added to the fermentation medium at the level of 1% as shown in Figure 2a. Extremely low production of alpha amylase (39.39 U/mg/min) was obtained with the addition of nutrient broth. It might be due to the fact that nitrogen limitations and requirements are very specific to the particular organism and vary with organisms. However, yeast extract gave maximum production of alpha amylase (67.34 U/mg/min) and protein content (548 µg/ml). Similar kinds of findings have also been reported by Hemilton et al. (1999). Thus, yeast extract

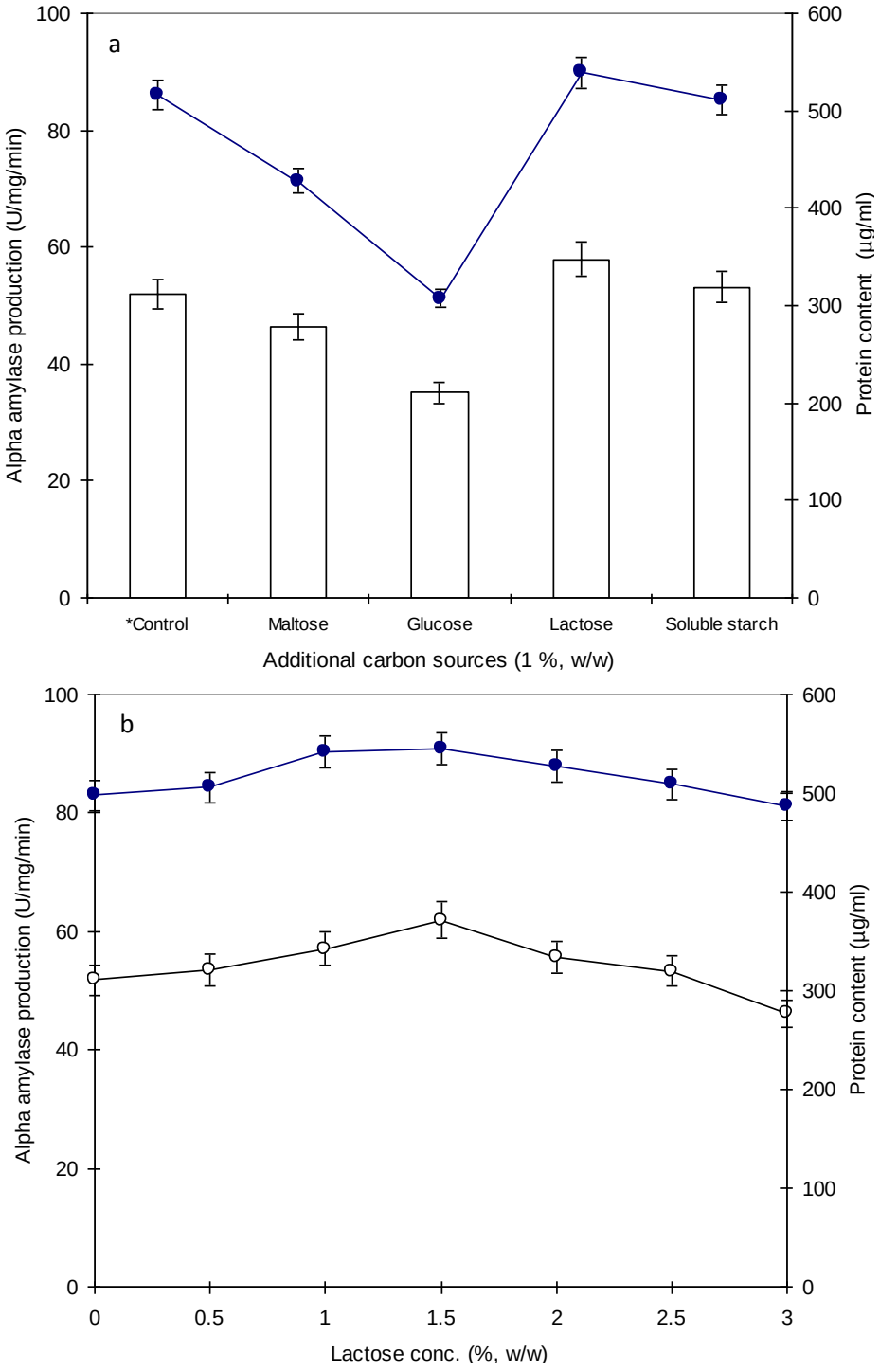

Figure 1. a) Effect of additional carbon sources; b) Effect of lactose concentration on the production of alpha amylase by *B. amyloliquefaciens* IIB-14 using solid state fermentation. Initial pH 7.2, temperature 40°C, incubation period 72 h, substrate to diluent ratio 1:1.25. Y-error bars indicate the standard deviation (±SD) among the values of three parallel replicates. -○-Alpha amylase production (U/mg/min), -●- Protein content (µg/ml).

was found to be optimal organic nitrogen source for the production of alpha amylase. The concentrations of yeast extract were varied from 0.5 to 3% as depicted in Figure 2b. Yeast extract (0.5%) resulted in low production of

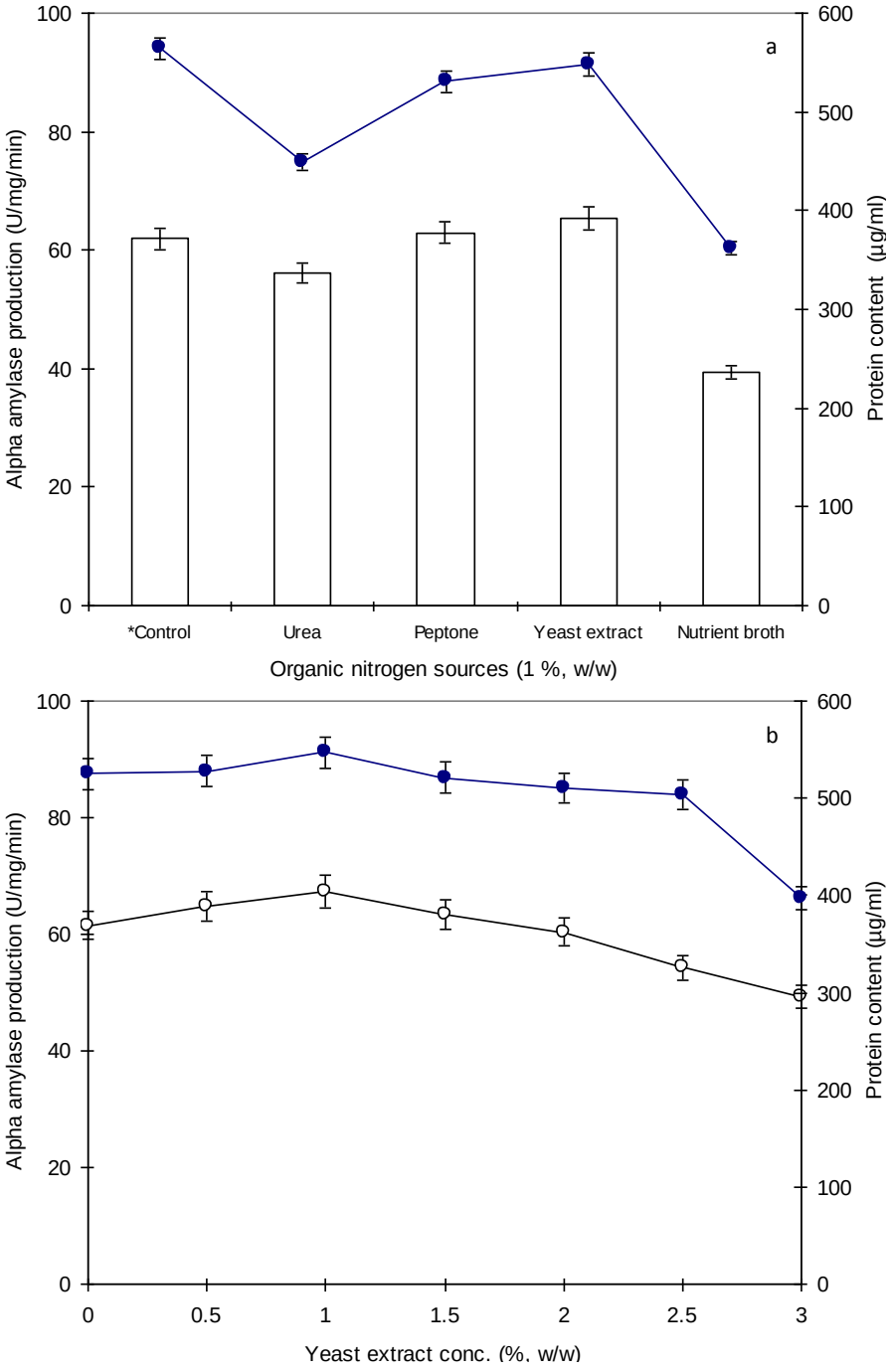

Figure 2. a) Effect of additional organic nitrogen sources; b) Effect of Yeast extract concentration on the production of alpha amylase by *B. amyloliquefaciens* IIB-14 using solid state fermentation. Initial pH 7.2, temperature 40°C, incubation period 72 h, substrate to diluent ratio 1:1.25. Y-error bars indicate the standard deviation (±SD) among the values of three parallel replicates. -○-Alpha amylase production (U/mg/min), -●- Protein content (µg/ml).

enzyme (64.84 U/mg/min). However, it was sharply increased with increase in the concentration of yeast extract from 0.5 to 1%. The yeast extract at the level of 1% gave optimal production of alpha amylase (67.32 U/mg/min) and protein content (547 µg/ml). By further increasing the level of yeast extract (1.5%), there was a marked decline in the production of enzyme. It was further decreased to 49.30 U/mg/min at the concentration

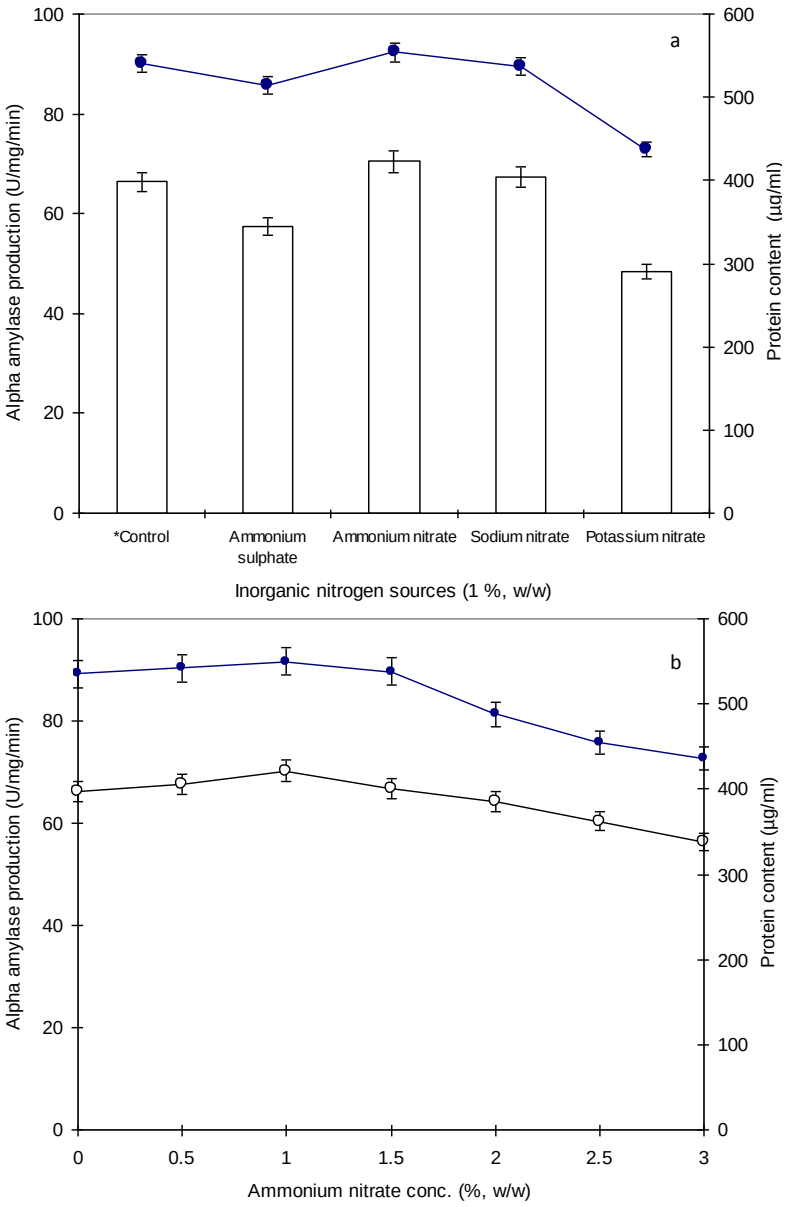

Figure 3. a) Effect of additional inorganic nitrogen sources; b) Effect of ammonium nitrate concentration on the production of alpha amylase by *B. amyloliquefaciens* IIB-14 using solid state fermentation. Initial pH 7.2, temperature 40°C, incubation period 72 h, substrate to diluent ratio 1:1.25. Y-error bars indicate the standard deviation (±sd) among the values of three parallel replicates. -○-Alpha amylase production (U/mg/min), -●- Protein content (µg/ml).

of 3%. Thus, the addition of 1% level of yeast extract was found to be optimal for further studies.

Different inorganic nitrogen sources such as $(NH_4)_2SO_4$, NH_4NO_3, $NaNO_3$ and KNO_3 were added to the fermentation medium at the level of 1% as shown in Figure 3a. Extremely low production of enzyme (48.30 U/mg/min) was obtained with KNO_3, probably due to the fact that nitrogen limitations and requirements are very specific to the particular organism and vary with organisms. However, NH_4NO_3 gave maximum production of alpha amylase (70.44 U/mg/min) and protein content (551 µg/ml). Similar kinds of findings have also been reported by Gangadharan et al., in 2006. Thus, NH_4NO_3 was found to be optimal inorganic nitrogen source for the production of alpha amylase. The concentrations of NH_4NO_3 were varied from 0.5 to 3% as highlighted in Figure 3b. NH_4NO_3 (0.5%) resulted in low production of enzyme (67.67 U/mg/min). However, it was sharply

Table 1. Application of Plackett-Burman design for alpha amylase productivity by *B*. *amyloliquefaciens* IIB-14.

Process parameters at two factorial design			Enzyme productivity (U/ml/min)	
Lactose (%) [A]	Yeast extract (%)[B]	Ammonium nitrate (%)[C]	Observed	Predicted
0.5	-	0.5	6.42	2.08
1	0.5	1	5.89	5.55
1.5	1	1	5.94	1.24
2	1	1.5	9.76	0.78
2.5	1.5	2	3.40	7.02

The letters (A, B and C) represent significant process parameters for fermentation.

Table 2. Statistical analysis of model at significant process parameters for alpha amylase productivity by *B*. *amyloliquefaciens* IIB-14.

Significant process parameters	Sum mean values	F value	Degree of freedom	Probability < p >
A	84.73	11.75	1	0.0561
B	0.346*E*+0025	16.02	3	0.0815
C	468.55	12.94	2	0.00978
Correlation	1.045*E*+0025			

CM, 16.92; *R*, 0.262.

increased with increase in the concentration of NH_4NO_3 from 0.5 to 1%. The NH_4NO_3 at the level of 1% gave maximum production of alpha amylase (70.15 U/mg/min) and protein content (550 µg/ml). By further increasing the level of NH_4NO_3 (1.5%), there was a sharp decline in the production of enzyme. It was further reduced to 56.46 U/mg/min at the concentration of 3%. In 1993, it was reported that high concentration of nitrogen has inhibitory effect on the production of alpha amylase (Yuguo et al., 1993). Thus, the addition of 1% level of NH_4NO_3 was found to be optimal for further studies.

In the fuzzy logic control system, production (*P*) was taken as controlled variable, operating parameters (*Q*) as manipulated variables and substrate concentration as changeable variable. The performance of the system using the specifically designed reaction vessel for alpha amylase productivity by *B. amyloliquefaciens* IIB-14 with input multiplicities in the operating variables was evaluated using the closed loop block diagram. Figure 5 highlights the sketch out model design. The change in error was worked out and converted to fuzzy form. The base rules were evaluated and the control input was calculated from the fuzzy logic. It was prepared by centroid method using Matlab and its related Simulink. It was further developed for the optimal reaction design using the fuzzy logic toolboxes. A suitable value for fuzzy implementation was also chosen. The process performance controlled by the fuzzy logic control system by the bacteria gave much better yield. The present design of reaction vessel led to a promising conversion rate of substrate into the final product.

The process parameters were determined using the Plackett-Burman design for alpha amylase productivity by *B*. *amyloliquefaciens* IIB-14 (Equations 1 and 2) and these data are given in Table 1. A statistical analysis of the responses for enzyme secretion was also performed and is shown in Table 2. Lactose as an additional carbon source was added to check the degree of freedom (dof) necessary for enzyme productivity. Analysis of linear, quadratic and interaction coefficients were undertaken on the carbon and nitrogen (both organic and inorganic) supplements. A slightly differential correlation between observed and predicted values was observed. The optimal levels for improved alpha amylase productivity in solid state culture were lactose as a carbon source (1.5%), yeast extract as an organic nitrogen source (1%) and ammonium nitrate as an inorganic nitrogen source (1%). The lower probability values and correlation of A, B and C values depicted that the model terms were significant ($p<0.05$). Ahuja et al. (2004) analyzed linear, quadratic and interaction coefficients and highlighted that enzyme secretion was a function of the independent parameters. The addition of ammonium nitrate as a potential N-source (degree of freedom=3) might have an important physiological role in enzyme stability as reported by Declerck et al. (2003).

The effect of pH on the activity of crude alpha amylase was studied as depicted in Figure 4a. The pH was varied from 5 to 7.5. The enzyme activity was extremely low at pH 5 (54.80 U/mg/min). However, it was sharply increased with increase in the pH from 5 to 5.5, and then gradually increased at pH 5.5 to 7.0. The activity of enzyme (71.24 U/mg/min) was found to be maximal at pH 7.0. Further increase in pH resulted in gradual decrease

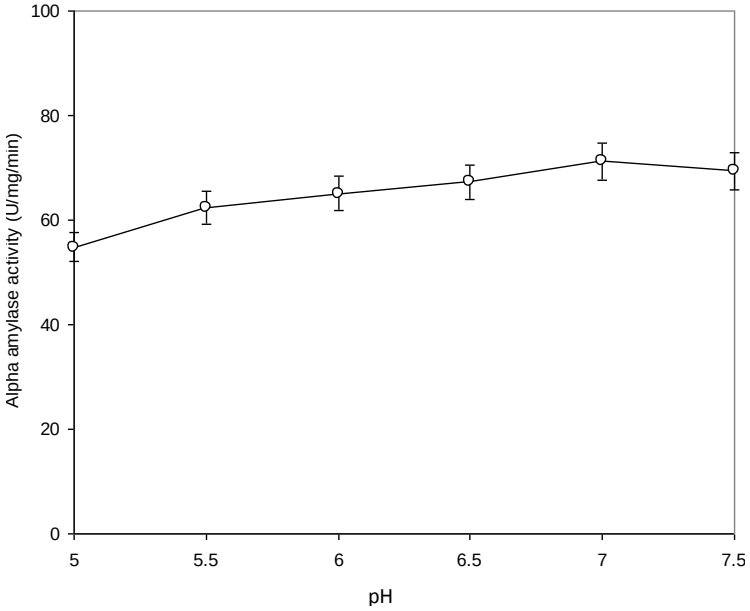

Figure 4a. Effect of pH on alpha amylase activity. Temperature 60ºC, incubation time 5 min. Y-error bars indicate the standard deviation (±SD) among the values of three parallel replicates. -○-Alpha amylase production (U/mg/min), -●- Protein content (µg/ml).

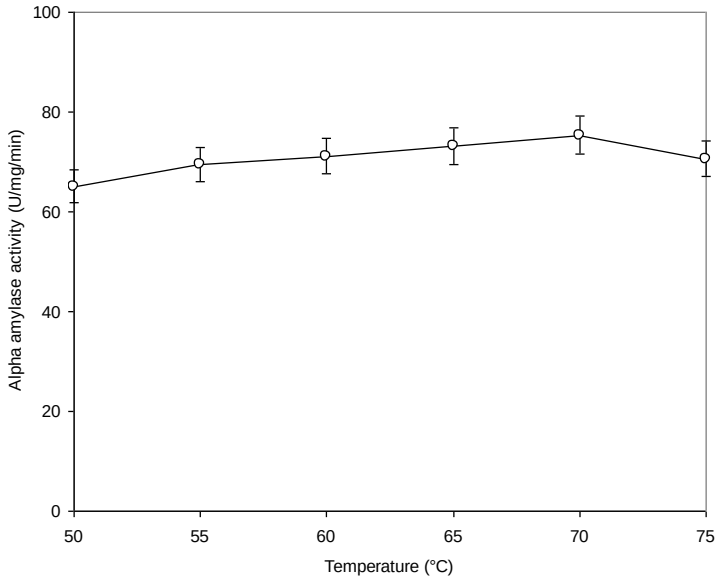

Figure 4b. Effect of Temperature on alpha amylase activity. pH 7.0, incubation time 5 min. Y-error bars indicate the standard deviation (±sd) among the values of three parallel replicates. -○-Alpha amylase production (U/mg/min), -●- Protein content (µg/ml).

in the enzyme activity. At pH 7.5, the activity of alpha amylase was decreased to 69.36 U/mg/min. Thus, pH 7 was found to be optimal for alpha amylase activity. The temperature was varied from 50 to 75°C as shown in Figure. 4b. The enzyme activity was extremely low at 50°C (65.04 U/mg/min). However, it was sharply increased with increase in the temperature from 50 to 55°C and then gradually increased at 55 to 70°C. The activity of enzyme (75.38 U/mg/min) was found to be maximal at 70°C. Further increase in temperature

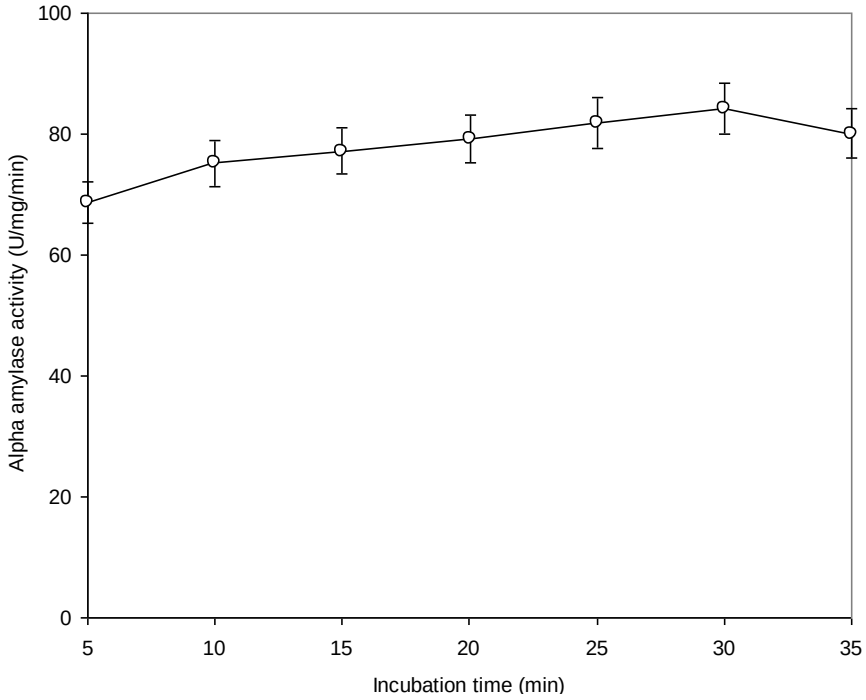

Figure 4c. Effect of incubation time on alpha amylase activity. pH 7.0, temperature 70ºC. Y-error bars indicate the standard deviation (±sd) among the values of three parallel replicates. -○-Alpha amylase production (U/mg/min), -●- Protein content (µg/ml).

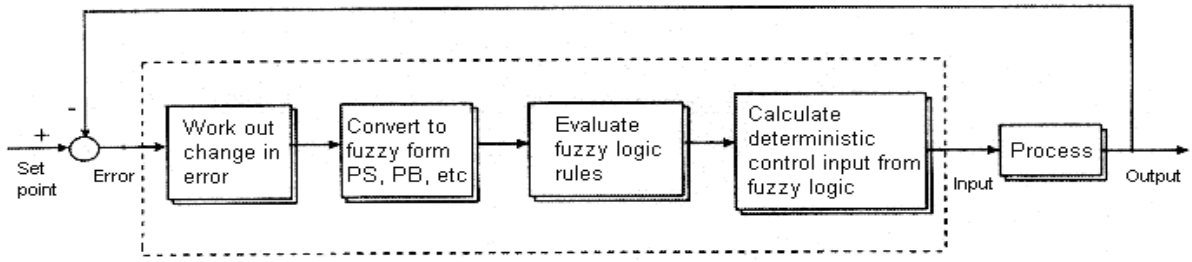

Figure 5. Design of the closed loop block diagram of fuzzy logic system for alpha amylase activity by *B. amyloliquefaciens* IIB-14 using solid state fermentation.

resulted in a sharp decline in enzyme activity. At 75°C, the activity of alpha amylase was decreased to 70.62 U/mg/min. Thus, 70°C was found to be optimal temperature for the activity of alpha amylase. Figure 4c depicts the effect of incubation time on the activity of alpha amylase. The incubation time was varied from 5 to 35 min. The enzyme activity was extremely low when it was incubated for 5 min (68.76 U/mg/min). However, it was sharply increased with increase in the time of incubation from 5 to 10 min and then gradually increased from 10 to 30 min. The maximum activity of enzyme (84.24 U/mg/min) was obtained by the incubation of enzyme for 30 min. Further increase in incubation time resulted in a sharp decline in enzyme activity. After 35 min of incubation, the activity of enzyme was decreased to 80.08 U/mg/min. Thus, incubation for 30 min was found to be optimal for the activity of alpha amylase. The decrease in alpha amylase activity at temperature, pH and incubation time other than optimal values is probably due to the denaturation/decomposition and inhibition of enzyme (Pandey et al., 2000; Declerck et al., 2003).

Conclusion

Studies on the effect of carbon and nitrogen sources on

the production and optimization of alpha amylase by *B. amyloliquefaciens* IIB-14 using solid state fermentation were carried out. On the basis of results obtained, conclusively, the enzyme productivity was enhanced using the optimal level of lactose (1.5%), yeast extract (1%) and NH_4NO_3 (1%). Fuzzy logic performance revealed highly promising substrate conversion rate of over 75%. Furthermore, the value of correlation (1.045*E*+0025) revealed that factorial terms were highly significant (*HS*, $p<0.05$). The alpha amylase showed maximum activity (84.24 U/mg/min) at pH 7 and temperature 70°C incubated for 30 min.

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