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Full Length Research Paper

Enhancement of alkaline protease production by *Bacillus clausii* using Taguchi experimental design

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The effect of culture conditions on protease production and bacterial growth of *Bacillus clausii* was investigated using Taguchi design of experiment. Five factors viz., carbon source, organic and inorganic nitrogen sources, agitation and metal ion, each at four levels were selected and an orthogonal array layout of L16 (4^5) were performed. The proposed medium for alkaline protease production consisted of (g/l): sucrose, 10; yeast extract, 10; KNO_3, 5; trace element without Mn^{2+}. Under these optimal conditions, 4 fold enhancement in protease production (from 250 to 1000 U/ml) was obtained. At the optimum culture for bacterial growth, which contained (g/l): starch, 10; yeast extract, 10; ammonium ions, 5; trace element without Zn^{2+}, 1.88 fold increase in growth production (from $OD_{600\ nm}$ of about 8.5 in basal medium to $OD_{600\ nm}$ of 16 in optimized medium) were observed. The inorganic nitrogen source was the most significant factor on protease production with 57.75% contribution. The organic nitrogen and carbon sources by 35.28 and 34.93% contributions were prominent factors in bacterial growth.

Key words: Alkaline protease, *Bacillus clausii*, medium optimization, taguchi method.

INTRODUCTION

Proteases constitute one of the most important groups of industrial enzymes, being extensively used in food, detergent, and other industries. Most of the available proteases produced commercially are of microbial origin. The alkaline proteases produced by *Bacillus* species are by far the most important group of enzymes produced commercially (Moon and Parulekar, 1991; Gupta et al., 2002).

It is well established that extracellular protease production in microorganisms is greatly influenced by media components. Therefore, the effect of various carbon and nitrogen nutrient cost-effective substrates, divalent metal ions, environmental and fermentation parameters such as pH, temperature, aeration, and agitation were evaluated in the literature (Adinarayana and Ellaiah, 2002; Varela et al., 1996). However, for optimization of all factors and establishment the best possible conditions by considering all interaction of parameters, numerous experiments have to be carried out, which is not economical and practical. For this type of cases, design of experiments (DOE) and statistical tools help to gain more information about the optimization conditions in a few trials (Krishna et al., 2005). There are two ways to dominate this problem may be addressed: conventional (classical) and statistical methods of DOE (Montgomery, 2001). Conventional optimization procedures involve altering of one parameter at a time keeping all other parameters constant, which enables one to assess the impact of those particular parameters on the process performance. These procedures are time consuming, cumbersome, require more experimental data sets and can not provide information about the mutual interactions of the parameters (Beg et al., 2003). Statistical experimental design methods have been widely employed in bioprocess optimization because these methods provide a systematic and efficient plan for experimentation under the consideration of the interactive effects among the control factors. Therefore, many control factors can be simultaneously studied and optimized (Abdel-Fattah et al., 2005; Rao et al., 2004). Among these, Taguchi method possesses the advantage that many factors can be examined simultaneously and much

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Table 1. Variables and their levels employed in the Taguchi's robust design method for optimal protease production by *B. clausii* in batch production.

Factors	Level 1	Level 2	Level 3	Level 4
Carbon source (1%)	Glucose	Glycerol	Starch	Sucrose
Organic nitrogen source (1%)	Peptone	Yeast	Casein	Casamino acids
Inorganic nitrogen source (0.5%)	$(NH_4)_2SO_4$	$NaNO_3$	KNO_3	NH_4Cl
Metal ion	$-Mn^{2+}$	$-Fe^{2+}$	$-Zn^{2+}$	$-Cu^{2+}$
Agitation (rpm)	100	200	300	350

quantitative information can be extracted by only a few experimental trials (Stone and Veevers, 1994; Houng et al., 2006). A few reports are available on the application of Taguchi's method in the field of biotechnology (Jeney et al., 1999; Cobb and Clarkson, 1994; Han et al., 1998). The basic principle of this method serves as screening filters which examine the effects of many process variables and identify those factors which have major effects on process using a few experiments (Dasu et al., 2003). Taguchi method of DOE involves establishment of large number of experimental situation described as orthogonal arrays (OA) to reduce experimental errors and to enhance their efficiency and reproducibility of the laboratory experiments. In the design of an OA, each column consists of a number of conditions depending on the levels assigned to each factor (Krishna Prasad et al. 2005).

This study is focused on formulation of a medium that substantially enhances synthesis and secretion of alkaline proteases in cultures of *B. clausii*. The series of experiments conducted to identify culture conditions that lead to improved protease production also enable investigation of the regulatory effects of important culture parameters including effect of different organic and inorganic nitrogen sources, different carbon sources, metal ions, and agitation on cell growth as well as protease production in this bacterium, which further facilitates economic design of the large scale fermentation operation system.

MATERIALS AND METHODS

Microorganism

The microorganism used in this study was isolated from indigenous soil samples, screened using a skim milk agar plate in alkaline broth. It was identified as *B. licheniformis* according to morphological and biochemical tests (Eftekhar et al. 2003). Further experiments based on partial sequence of 16S rRNA homology showed that it was closely related to *B. clausii* (Hesampour et al., 2006).

Culture maintenance

Stock cultures of the isolate were stored in 30% glycerol at –70°C. Prior to each experiment, the bacterium was subcultured from the frozen stocks onto a solid alkaline medium as basal medium (pH 10.5) containing (g/l): glucose, 10; peptone, 5; yeast extract, 5; KH2PO4, 1; $MgSO_4.7H_2O$, 0.2; Na_2CO_3, 10; Agar, 15 (Takami et al., 1989).

Seed culture medium

For enzyme production, bacterial cells from a 24 h aged culture were inoculated into 250 ml Erlenmeyer flasks containing 50 ml of sterile inoculation medium. The composition of the inoculum medium was the same as described for culture maintenance. The cultures were grown at 35°C on a stirrer at 350 rpm for 16 h. After reaching an optical density of about 2.5 at 600 nm, 3% (v/v) of the culture was used to inoculate production flasks.

Cultivation medium and culture conditions

According to Taguchi's OA, orthogonal array sixteen experiments were used to evaluate the effect of five variables which showed significant influence on the enzyme production in four levels (Table 1). The decision on the concentrations of these components was based on literature data used for these types of fermentations (Adinarayana et al., 2002; Beg et al., 2003; Tari et al., 2006; Joo et al., 2002; Chauhan and Gupta, 2004).

Experiments were performed according to an experimental plan given in the Table 2. The composition of the media was varied according to the experimental plan given in Table 2.

The composition of the constant constituents of the media consists (g/l): K_2HPO_4, 4; trisodium citrate, 4; $CaCl_2$, 0.002; $MgSO_4.7H_2O$, 0.5; Na_2CO_3, 10. After autoclaving and cooling the medium, 10 ml of a trace element solution containing (g/l): trisodium citrate, 10; $(NH_4)_6MO_7O_{24}$, 0.1; $MnSO_4.H_2O$, 0.5; $FeSO_4.7H_2O$, 2; $CuSO_4.5H_2O$, 0.2; $ZnCl_2$, 0.2, were added to one liter of the medium. In order to study the effects of different elements on protease production, the reference media was enriched with addition of a trace element solution that one of its elements including Mn^{2+}, Zn^{2+}, Fe^{2+},and Cu^{2+} is eliminated from the solution and the other remaining elements were added to the media. Cultures were incubated at 35°C, with different agitations (rpm). The initial pH of the medium was adjusted to 10.0 with sterile Na_2CO_3 solution and was not controlled during the course of fermentation.

Optimization methodology

In this experimental design a standard orthogonal array L16 (4^5) with 15 degree of freedom was used to examine four factors in five levels. The L and the subscript (16) represent the Latin square and the number of experimental runs, respectively. The levels of the factors studied and the layout of the L16 Taguchi's OA, orthogonal array are shown in Tables 1 and 2.

The experimental results were analyzed to extract independently the main effects of the factors; the analysis of variance technique was then applied to determine which factors were statistically significant. The controlling factors were identified, with the magnitude of effects qualified and the statistically significant effects determined. Accordingly, the optimal conditions were determined by combining the levels of factors that had the highest main effect value. All calculations were performed using Design Expert software (version

Table 2. L16 (4^5) orthogonal array of Taguchi design of experiments and corresponding alkaline protease production by *Bacillus clausii* in batch production.

Trial No.	A	B	C	D	E	OD_{600} (nm)	Activity (U/ml)
1	1	1	1	1	1	4.0±0.1	70±15
2	1	2	2	2	2	6.5±0.2	680±27
3	1	3	3	3	3	3.0±0.1	366±20
4	1	4	4	4	4	7.0±0.25	110±15
5	2	1	2	3	4	1.5±0.1	328±18
6	2	2	1	4	3	4.8±0.2	100±16
7	2	3	4	1	2	3.2±0.15	47±13
8	2	4	3	2	1	3.5±0.1	625±11
9	3	1	3	4	2	4.5±0.1	544±22
10	3	2	4	3	1	16.0±0.5	273±6
11	3	3	1	2	4	7.5±0.2	274±19
12	3	4	2	1	3	5.8±0.1	717±22
13	4	1	4	2	3	7.0±0.2	190±17
14	4	2	3	1	4	9.0±0.25	1000±20
15	4	3	2	4	1	3.8±0.1	170±14
16	4	4	1	3	2	7.5±0.2	230±15

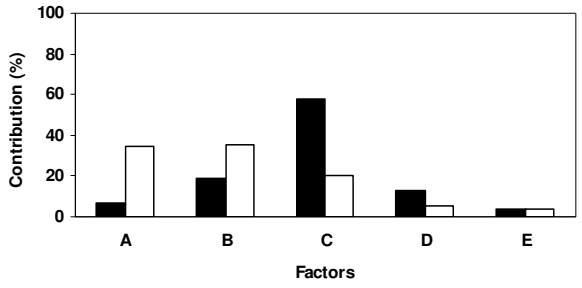

Figure 1. Contribution of five factors on protease production (■) and bacterial growth (□) by *B. clausii*.

6.0.10, Stat-Ease Inc., USA).

Assays

The proteolytic activity of the enzyme was determined using casein as a substrate. Casein was dissolved in 0.1 M Tris-HCl buffer (pH 9.0) at a concentration of 1%. The assay mixture consisted of 450 µl of substrate and 50 µl of enzyme solution suitably diluted with 0.1 M Tris-HCl buffer (pH 9.0). The reaction mixture was incubated at 35°C for 10 min and the reaction was terminated by the addition of 500 µl of 10% trichloroacetic acid (TCA), and then centrifuged at 5000×g for 15 min to remove the resulting precipitate. Protease activity was determined as released tyrosine from the supernatants. One unit of enzyme activity was defined as the amount of the enzyme resulting in the release of 1 µg of tyrosine per min at 35°C under the reaction conditions (Joo et al., 2002). Growth content was evaluated by spectrophotometer as optical density at 600 nm.

RESULTS

Experimental design is the sequence of steps initially taken to ensure that the data will be obtained in such a way that its analysis will lead immediately to valid statistical inferences. Experiments study with the designed experimental condition showed significant variation in the protease activity (Table 2). Protease production was found to be greatly dependent on the culture conditions.

Figure 1 presents the contribution of selected factors on the growth and protease production. It can be observed that inorganic nitrogen source with 57.75% and organic nitrogen source with 35.28% have shown highest positive impact on the protease production and growth, respectively. Organic nitrogen and carbon sources are also significant factors on the enzyme production and bacterial growth with 18.83 and 34.93%, respectively. Agitation showed least impact among the factors studied with the assigned variance of values. Therefore, the analysis of variance (ANOVA) for two responses of protease production and bacterial growth were carried out according to the factors with the contribution more than 10% as suggested by software.

In Taguchi approach, ANOVA is used to analyze the results of the OA experiments and determine how much variation of each factor has contributed. By studying the main effects of each of the factors, the general trends of the influence of the factors towards the process can be characterized. The characteristics can be controlled such that a lower or a higher value in a particular influencing factor produces the preferred result. Thus, the levels of factors to produce the best results can be predicted (Krishna Prasad et al., 2005).

Analysis of the data for the determination of significant parameters on protease production and bacterial growth has been performed and the results are shown in ANOVA Tables 3 and 4. From the calculated ratios (F), it can be referred that the factors considered in the experimental

Table 3. ANOVA for alkaline protease production by *B. clausii*.

Source	Sum of squares	DF	Mean square	F-Value	Prob>F
Model	1.031E+006	9	1.145E+005	5.58	0.0243*
B	2.172E+005	3	72396.83	3.53	0.0882*
C	6.662E+005	3	2.221E+005	10.83	0.0078*
D	1.471E+005	3	49038.17	2.39	0.1673
Residual	1.230E+005	6	20502.92		
Total	1.154E+006	15			

B: Organic nitrogen source; C: Inorganic nitrogen source; D: Metal ions; * significant term.

Table 4. ANOVA table for bacterial growth by *Bacillus clausii*.

Source	Sum of squares	DF	Mean square	F-Value	Prob>F	
Model	155.73	9	17.30	6.57	0.0163*	Significant
A	59.92	3	19.97	7.58	0.0183*	
B	60.52	3	20.17	7.66	0.0178*	
C	35.29	3	11.76	4.47	0.0567*	
Residual	15.81	6	2.63			
Total	171.54	15				

A: Carbon source; B: Organic nitrogen source; C: Inorganic nitrogen source; * significant terms.

Table 5. Point prediction for optimum conditions of growth and protease production.

	Prediction	SE Mean	95% CI Low	95% CI High	Optimum conditions
Optical Density	14	1.28	10.86	17.14	A: 3; B: 2; C: 4; D: Ns; E: Ns
Protease Activity (U/ml)	890	113.20	613.01	1166.99	A: Ns; B: 2; C: 3; D: 1; E: Ns

SE: Standard Error, CI: Confidence Interval, (A-E): factors, (1-4): Levels, Ns: Not specific level.

design are statistically significant at 95% confidence limit.

The ANOVA of protease production has the model *F*-value of 5.58 that implies the model is significant. The model obtained from ANOVA indicated that the multiple correlation coefficient of R^2 is 0.8934 i.e. the model can explain 89.34% variation in the response. Also, the model has an adequate precision value of 7.058; this suggests that the model can be used to navigate the design space. The "adequate precision value" is an index of the signal to noise ratio and a value >4 is an essential prerequisite for a model to be a good fit. The model shows standard deviation, mean, C.V. and predicted residual sum of square (PRESS) values of 143.19, 357.75, 40.02 and 8.748E + 005, respectively.

The ANOVA of bacterial growth (Table 4) has the model *F*-value of 6.57 that implies the model is significant, and its R^2 is 0.9079 i.e. the model can explain 90.79% variation in the response. The model has an adequate precision value of 10.853 and has standard deviation, mean, C.V. and PRESS values of 1.62, 5.91, 27.45 and 112.39, respectively. The main effect plots of the model graph clearly depict the optimum level of each significant factor on protease production (Figure 2). The one factor or main effect plots of the model graph for optimum levels of significant factors on bacterial growth is depicted in Figure 3. Point prediction for achieving highest bacterial growth and protease production in terms of levels of factors shown in Table 5. Under optimal conditions for alkaline protease, the expected activity was 890 U/ml. The predicted optical density (OD_{600} nm) was 14 under optimal condition of growth (Table 5).

Furthermore, to validate the proposed experimental methodology, fermentation experiments were repeatedly performed for protease production and bacterial growth by employing the obtained optimized culture conditions. The results compared with growth and protease production in basal medium and depicted in Figure 4.

DISCUSSION

In the present work, the cultivation conditions for bacterial growth and protease production of *B. clausii* in shake flask cultures by the Taguchi experimental method were

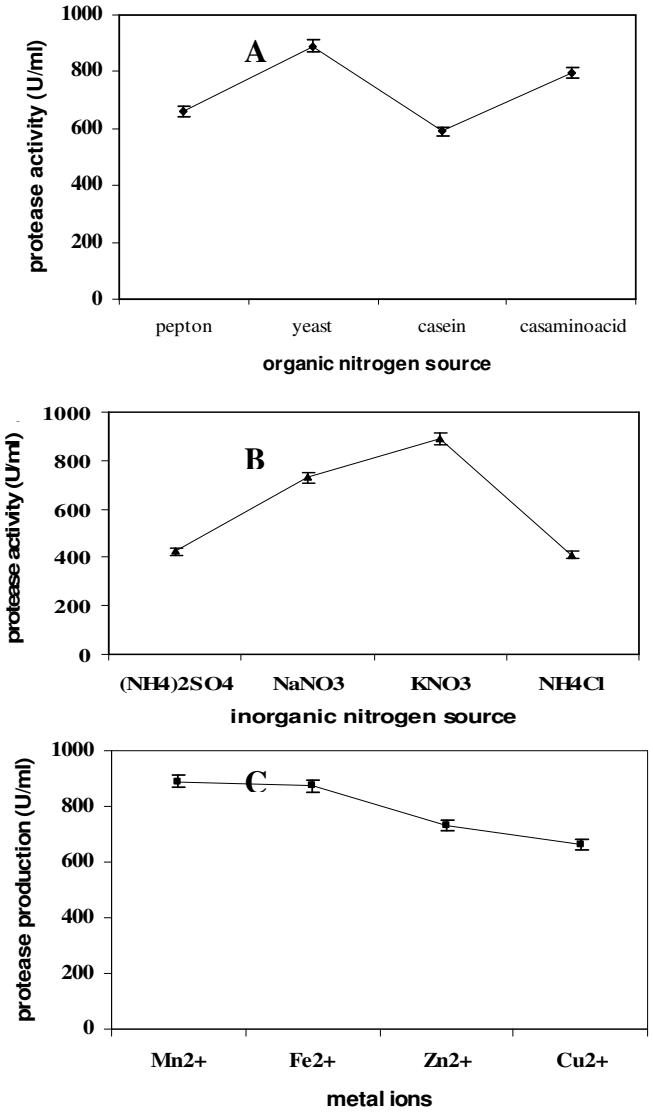

Figure 2. Variation in protease production according to different levels of (A) organic nitrogen source, (B) inorganic nitrogen source, and (C) metal ion. Two other factors of carbon source and agitation have no significant contributions on protease production.

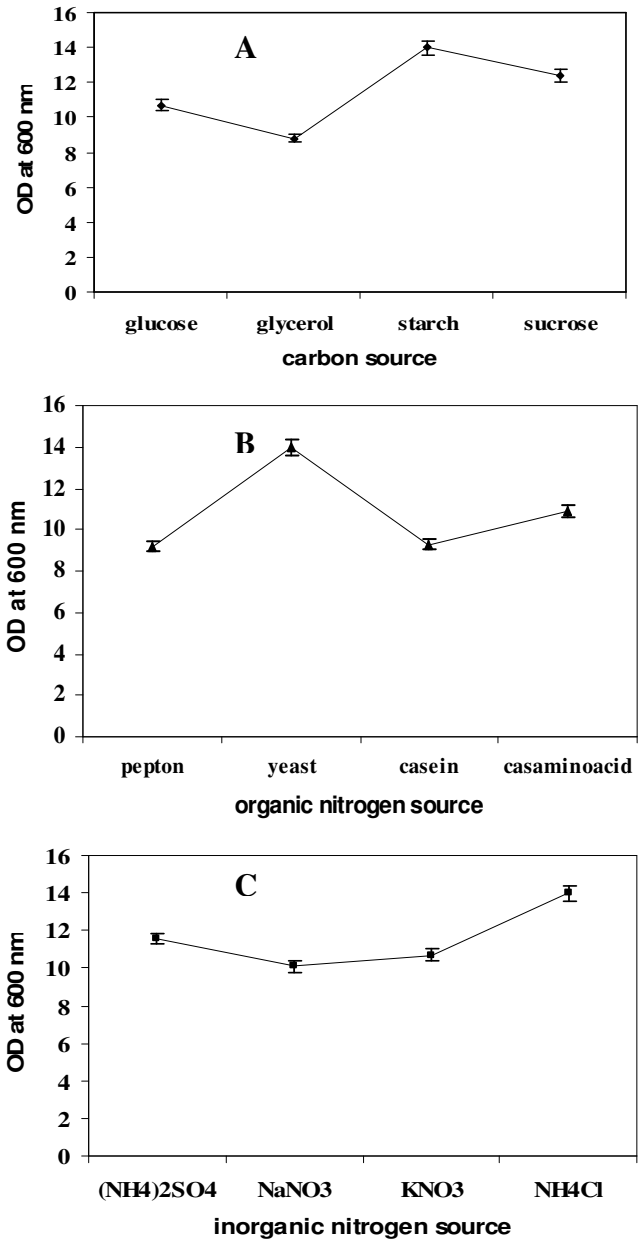

Figure 3. Variation in bacterial growth according to different levels of (A) carbon, (B) organic nitrogen, and (c) inorganic nitrogen sources. Two other factors of metal ion and agitation have no significant contributions on cell growth.

studied. We have considered a set of culture parameters that substantially enhance protease production and bacterial growth. In the process, the effects of important culture parameters such as organic and inorganic nitrogen source, carbon source, aeration rate and metal ions on protease production and cell growth were investigated.

Table 3 indicated that the highest protease activity was achieved in the presence of yeast extract and potassium nitrate as organic and inorganic nitrogen sources. On the basis of ANOVA, there are no difference between the levels of carbon source, metal ion and agitation inthis case; therefore, theoretically there is no significant difference in the selection of their levels in optimum protease production. However, *B. clausii* produced maximum alkaline protease in the presence of sucrose and yeast extract (i.e. 1000 U/ml), which is clearly resulted in trial No.14 of Table 2.

Analysis of Variance of data for bacterial growth (Table 4) showed that the optimum bacterial growth was obtained in presence of starch, yeast extract and ammonium chloride as carbon, organic nitrogen and inorganic nitrogen sources. There was no significant difference between metal ion and agitation levels. Because *B. clausii* is an aerobic bacterium and for reducing the time of the pro-

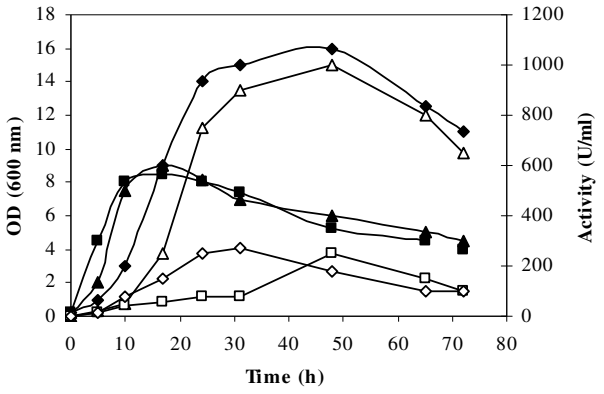

Figure 4. Time profiles of protease production (empty symbols) and bacterial growth (filled symbols) by using *B. clausii* in the basal medium (squares), the optimized protease production medium (triangles) and the optimized growth medium (diamonds).

cess, it seems that the highest level of aeration rate of 350 rpm during growth phase was applied, which has no adverse effect on bacterial growth.

The suggested culture for bacterial growth contains (g/l): starch, 10; yeast extract, 10; ammonium ions, 5; trace element solution without Zn^{2+} and with 350 rpm agitation that is similar to the conditions which were used in trial number 10. The proposed medium for alkaline protease production contains (g/l): sucrose, 10; yeast extract, 10; KNO_3, 5; trace element without Mn^{2+} and with 350 rpm rotation speed that is like to the medium number 14 in Table 2.

The experimental data showed four fold improvement in protease production (from 250 U/ml in basal medium to 1000 U/ml in optimized medium), and 1.88 fold increase in bacterial growth (from $OD_{600\ nm}$ about 8.5 in basal medium to $OD_{600\ nm}$ of 16 in optimized medium).

As it was well documented in the literature, the culture conditions that promote production of enzymes like proteases are significantly different from the culture conditions promoting cell growth (Moon and Parulekar, 1991). In this regard, this paper proposes two low cost media formulations that could be of industrial value for protease production and bacterial growth and could serve as basal media for further optimization studies of this and similar strains. Therefore, it is suggested to separate growth phase from protease production phase in order to further enhance protease synthesis from *B. clausii*.

These experiments provided basic information to improve the efficiency of protease production, and supported the analysis of the main effect of each constituent of the medium. Therefore, this study serves as another example for the application of the Taguchi methodology for improvement of biological processes.

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