Taguchi experimental design for medium optimization for enhanced protease production by *Bacillus subtilis* HB04

C.K. Venil[1] and P. Lakshmanaperumalsamy[2]

[1]Division of Environmental Microbiology, Department of Environmental Sciences, Bharathiar University, Coimbatore – 641 046, Tamil Nadu, India.
[2]Karpagam University, Coimbatore – 641 021, Tamil Nadu, India.
Email: ckvenil@gmail.com; drplpsamy@gmail.com

Abstract

Bacillus sp. is specific producers of proteases amongst bacteria and protease enzymes are of significant ones due to their multifarious applications. In this research,Taguchi experimental design was applied to optimize the conditions for protease production by *Bacillus subtilis* HB04. This approach facilitated the study of interaction of a large number of variables spanned by factors and their settings with a small number of experimental runs leading to considerable economy in time and cost for the process optimization. The objective of this research was to determine the significant parameters in the production of protease by *Bacillus subtilis* HB04 in submerged fermentation. Five factors viz., carbon, nitrogen, metal ions, temperature and agitation, each at four levels were selected and an orthogonal array layout of L_{16} (4^5) performed. The experiment result indicated that dextrose (1 %), $(NH_4)_2PO_4$ (0.5 %), temperature (30^{o}C) and agitation (100 rpm) were the important factors for protease production by *Bacillus subtilis* HB04 in submerged fermentation at the optimum levels.

Keywords: *Bacillus subtilis* HB04; Protease; Taguchi design; contribution

INTRODUCTION

Microbial enzymes are more advantageous than enzymes derived from plants or animals because of their great variety of catalytic activities, possible high yields, stability, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations, rapid growth of microorganisms in inexpensive media, more convenient and safer protection methods (Hasan *et al.*, 2006). Only about 2% of the world's microorganisms have been tested as enzyme sources. Bacterial strains are generally more used as they offer higher activities compared to yeasts (Frost and Moss, 1987) and tend to have neutral or alkaline pH optima and are often thermostable.

Of the microbial enzymes, alkaline protease is of particular interest due to its primary applications in the detergent industry since its introduction in the 1914 as a cleaning additive (Erikson, 1996; Anwar, 1998; Gupta *et al.*, 2002). Proteases account for approximately 60% of all enzyme sales because of their varied applications in food, pharmaceutical and number of other industries (Ikasari and Mitchell, 1996) such as leather industry, manufacture of protein hydrolizates and waste processing industry (Pastor *et al.*, 2001). Alkaline protease added to laundry detergents plays a specific catalytic role in the hydrolysis of protein strains such as blood, milk, human sweat,

etc. The increased usage of the protease as a detergent additive is mainly due to its environmentally acceptable cleaning capabilities.

Amongst bacteria, *Bacillus* sp. is specific producers of proteases (Priest, 1977). It is a well known fact that extracellular protease production in microorganisms is greatly influenced by media components, especially carbon and nitrogen sources (Hanlon *et al.*, 1982; Kole *et al.*, 1988a; Kole *et al.*, 1988b; Kaur *et al.*, 2001), metal ions (Varela *et al.*, 1996) and physical factors such as pH, temperature, inoculum density (Nehete *et al.*, 1985), dissolved oxygen (Moon and Parulekar, 1993) and incubation time (Nehete *et al.*, 1985; Oberoi *et al.*, 2001). 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 and reported in the literature that optimum conditions of medium factors are required for maximum enzyme production (Varela *et al*., 1996; Adinarayana and Ellaiah, 2002). 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 cannot provide information about the mutual interactions of the parameters (Beg *et al*., 2003).

Alternative to conventional optimization procedures, design of experiments (DOE) and statistical tools help to gain more information about the optimization conditions in a few trials (Krishna *et al.*, 2005). Statistical experimental design methods provide a systematic and efficient plan for bioprocess optimization considering the interactive effects among the control factors. Many control factors can be simultaneously studied and optimized by statistical experimental designs (Rao *et al*., 2004; Abdel-Fattah *et al*., 2005).

Among various statistical experimental designs, Taguchi experimental design offers distinct advantages by which many factors can be examined simultaneously and much quantitative information can be extracted with 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 (Cobb and Clarkson, 1994; Han *et al.*, 1998; Jeney *et al*., 1999). 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 (Krishna Prasad *et al.*, 2005).

In this study, Taguchi method was applied to test the relative importance of medium components (i.e., carbon, nitrogen and metal ions) and environmental factors (i.e., temperature and agitation speed) in protease production by *Bacillus subtilis* HB04, an enteric gut associated microflora of honey bee (*Apis mellifera*), a new source for microbial exploitation. This small investigation was undertaken to test the potential of insect enteric gut associated bacterium and to optimize the production condition.

MATERIALS AND METHODS

Microorganisms and media

The bacterium *Bacillus subtilis* HB04 was isolated from enteric gut of honey bee (*Apis mellifera*) and the culture was maintained at 4^{o}C and subcultured every two weeks. Nutrient broth medium containing (g/l): Beef extract: 3.0 g; Yeast extract: 3.0 g; NaCl: 5.0 g; Peptone: 5.0 g was prepared for the production of protease from *Bacillus subtilis* HB04. The pH of the medium was adjusted to 7.0 with 1N NaOH or 1 N HCl and was autoclaved at 121°C for 15 minutes.

Production of protease

100 ml of nutrient broth was inoculated with 1 ml of the inoculum (containing 10^6 cells/ml) and was incubated at 30°C for 18 hours. After incubation the crude enzyme was obtained by centrifugation of the culture broth at 10000×g for 10 minutes at 30°C. The cell free supernatant which contains the enzyme was assayed for protease activity.

Enzyme assay

Protease production was assayed in terms of protease activity exhibited by the culture supernatant in the enzyme assay. Protease assay was done by a modification of the casein digestion method of Kunitz (1947). To 3 ml of 0.6% casein in a phosphate buffer (100 mM) of pH 7, 0.5 ml of crude enzyme was added and incubated for 30 minutes at 37°C after which 3 ml of 5% trichloroacetic acid was added to stop the reaction and allowed to stand for 15 minutes at room temperature. The resultant mixture was filtered through Whatman No. 1 filter paper. The absorbance of this filtrate was measured at 280 nm in a UV Visible spectrophotometer. A suitable control was run simultaneously, in which Trichloroacetic acid (TCA) was added prior to the addition of enzyme solution. One unit of proteolytic activity was defined as that amount of enzyme, which liberated 1 µg of tyrosine (Sigma-Aldrich) per ml per minute under the specific conditions of assay. The absorbance at 280 nm (test-control) indicated the tyrosine content of the filtrate, which has been released by the hydrolysis of the protein substrate by the enzyme. The tyrosine content of the sample was read from the standard calibration curve prepared with pure tyrosine.

Optimization methodology

Taguchi experimental design, a standard orthogonal array L_{16} (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 L_{16} Taguchi's 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 7.1.5, Stat-Ease Inc., USA).

Table 1: Factors and their levels employed in the Taguchi's experimental design for protease production by *Bacillus subtilis* HB04

Factors	Level 1	Level 2	Level 3	Level 4
Carbon source (1 %)	Dextrose	Glucose	Lactose	Sucrose
Nitrogen source (0.5 %)	NH_4Cl	NH_4NO_3	$(NH_4)_2PO_4$	$NaNO_3$
Metal ion (0.02 %)	Ca^{2+}	Cu^{2+}	Fe^{2+}	Mn^{2+}
Temperature (^oC)	20	25	30	35
Agitation (rpm)	0	50	100	200

Table 2: L_{16} (4^5) orthogonal array of Taguchi experimental design and corresponding protease production by *Bacillus subtilis* HB04

Run	A:Carbon (1 %)	B:Nitrogen (0.5 %)	C:Metal ions (0.02 %)	D:Temperature (^oC)	E:Agitation (rpm)	Protease (U/mL)
1	3	4	2	1	3	98.9
2	2	2	1	4	3	289.51
3	1	1	1	1	1	112.16
4	4	1	4	2	3	34.51
5	3	1	3	4	2	152.24
6	4	4	1	3	2	101.23
7	1	2	2	2	2	80.31
8	4	3	2	4	1	183.32
9	2	4	3	2	1	109.38
10	1	4	4	4	4	140.49
11	3	2	4	3	1	90.21
12	3	3	1	2	4	179.32
13	2	3	4	1	2	168.3
14	1	3	3	3	3	155.72
15	2	1	2	3	4	120.38
16	4	2	3	1	4	89.11

RESULTS

Taguchi experimental design is a good positive option for the optimization of biotechnological processes for production of microbial enzymes. In this case, the influence of 5 factors on the protease production by *Bacillus subtilis* HB04 was tested in Taguchi experimental design in 16 runs. The results of Taguchi experimental design in 16 runs, for the five factors, i.e., carbon, nitrogen, metal ions, temperature and agitation chosen for optimization of protease production by the strain *Bacillus subtilis* HB04 (Table 2) show the efficiency of protease production ranging from 34.51 – 289.51 U / mL corresponding to the combined effect of the five factors in their specific ranges. The experimental results suggest that these factors at optimum level strongly support the production of protease.

In run 4, sucrose (1 %), NH_4NO_3 (0.5 %), Fe (0.02 %), temperature (20^oC) and agitation (200 rpm), lowest production of 34.51 U/ mL was observed. The production of 289.51 U / mL was observed in run 2 with a combination of Dextrose (1 %), $(NH_4)_2PO_4$ (0.5 %), Fe (0.02 %), temperature (30^oC) and agitation (100 rpm). Figure 1 presents the contribution of selected factors on the protease production. It can be observed that carbon, agitation, nitrogen and temperature respectively contributing of 33.47, 23.53, 19.71 and 15.24 % have shown highest positive impact on the protease production. Metal ions showed least impact among the factors studied with the assigned variance of values. Therefore, the analysis of variance (ANOVA) for the responses of protease production was carried out according to the factors which contributed more than 10% as suggested by the Taguchi method.

In Taguchi approach, ANOVA is used to analyze the results of the OA experiments and determine how much variation that 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 distinguished.

Analysis of the data for the determination of significant parameters on protease production was performed and the results are shown in ANOVA Tables 3. From the calculated ratios (F), it can be inferred that the factors considered in the experimental design are statistically significant at 95% confidence limit. The ANOVA of protease production has the model F value of 81.5278 that implies the model is significant. The model obtained from ANOVA indicated that the multiple correlation coefficient of R^2 is 0.9969 i.e. the model can explain 99.69% variation in the response. Also, the model has an adequate precision value of 9.286; 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 7.20, 131.57, 5.47 and 424.89 respectively. Point prediction for achieving highest protease production in terms of levels of factors is shown in Table 4. Under optimal conditions for protease, the expected activity was 290 U/mL.

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.

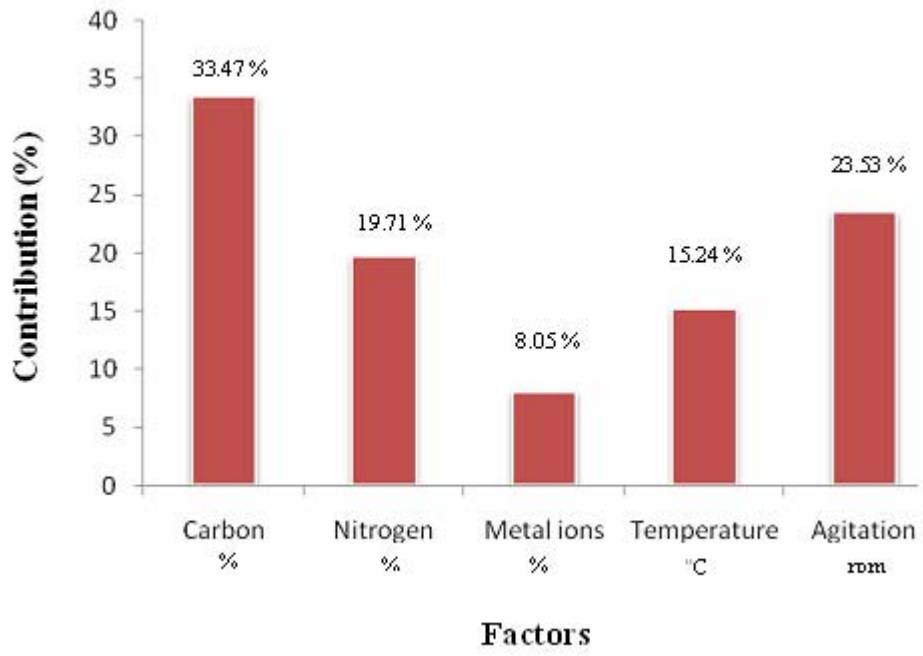
Table 3: ANOVA for protease production by *Bacillus subtilis* HB04

Source	Sum of squares	DF	Mean square	F value	Prob > F	
Model	50730.7	12	4227.56	81.5278	0.0020	significant
A-Carbon	31153.3	3	10384.4	200.263	0.0006	
B-Nitrogen	6008.28	3	2002.76	38.6229	0.0068	
D-Temperature	2516.5	3	838.832	16.1767	0.0234	
E-Agitation	11052.6	3	3684.18	71.0489	0.0028	
Residual	155.563	3	51.8542			
Cor Total	50886.2	15				

Table 4: Point prediction for optimum conditions of protease production by *Bacillus subtilis* HB04

Prediction	SE Mean	95 % CI Low	95 % CI High	Optimum conditions
290.	6.49	139.59	179.51	Dextrose (1%), $(NH_4)_2PO_4$ (0.5%), temperature (30^oC) and agitation (100 rpm)

Figure 1: Contribution of five factors on protease production by *Bacillus subtilis* HB04 in a submerged culture using Taguchi experimental design



DISCUSSION

Each organism has its own special conditions for maximum enzyme production. Statistical methods have been applied for optimization of microbial enzyme production in various studies (Dey *et al.*, 2001; Francis *et al.*, 2002; Ahuja *et al.*, 2004; Kunamneni *et al.*, 2005). The use of a good reliable statistical model is essential to develop better strategies for the optimization of the fermentation process (Ghaley *et al.*, 2005). In this regard, Taguchi approach of orthogonal array experimental design for bioprocess optimization is a good reliable statistical model. Seyedeh *et al.* (2007) applied Taguchi experimental design for alkaline protease production by *Bacillus clausii* and reported that this experiment design provided basic information to improve the efficiency of protease production and also supported the analysis of the main effect of each constituent of the medium and concluded that his study would serve as another example for the application of the Taguchi methodology

for improvement of biological processes. Heravi *et al.* (2008) also successfully applied Taguchi experimental design for lipase production by *Bacillus* F3 and observed enhanced production.

Taguchi experimental design involves a study of given system by a set of independent variables (factors) over a specific region of interest (levels) by identifying the influence of individual factors, establishing the relationship between variables and also the performance at the optimum levels. By studying the main effects of each of the factors, the general trends of the influence of the factors towards the process can be predicted and 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 (Sreenivas Rao *et al.*, 2004; Chang *et al.*, 2006). Any individual factor may interact with the other factors creating the possibility of presence of interactions. This kind of interaction is possible in Taguchi design of experiment. Estimated interaction severity index (SI) of the factors under study helps to know the influence of two individual factors at various levels of the interactions (Han *et al.*, 1998; Venkata Dasu *et al.*, 2003; Koo *et al.*, 2006).

In this study, protease production by *Bacillus subtilis* HB04 in submerged fermentation, dextrose (1 %) as carbon source, $(NH_4)_2PO_4$ (0.5 %) as nitrogen source, temperature (30^oC) and agitation (100 rpm) up to level 5, constituted the main factors of the medium. The contribution of five factors in protease production by Taguchi experimental design showed that carbon source played a leading role than the other selected parameters (carbon33.47 %, agitation25.53 %, nitrogen 19.71 %, temperature 15.24 % and metal ions 8.90 %). Point prediction of the design showed that maximum protease production of 290 U / mL was achieved under optimal experimental conditions. This result would further facilitate economic design of the large scale fermentation operation system.

CONCLUSIONS

Taguchi design was successfully applied to test the relative importance of medium components and environmental factors on protease production. The selected orthogonal array was L_{16} and optimum factors for protease production were found to be dextrose, $(NH_4)_2PO_4$, temperature and agitation. The optimum medium condition derived was: dextrose (1 %), $(NH_4)_2PO_4$ (0.5 %), temperature (30^oC) and agitation (100 rpm). At this optimum condition, the yield of protease production by *Bacillus subtilis* HB04 was found to be 290 U / mL.

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