JOURNAL OF PURE AND APPLIED MICROBIOLOGY, May 2015. Vol. **9**(Spl. Edn. 1), p. 475-481

Process Optimization of Organophosphate Hydrolase Production by using *Brevibacillus parabrevis* SR2729

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(Received: 19 October 2014; accepted: 02 January 2015)

Organophosphate hydrolase enzyme has been reported to efficiently degrade organophosphate compounds. Thus its increased demand, has led us to improve its production by process optimization. Statistical improvement for OPH production by Response Surface Methodology (RSM) was performed by using the bacterial strain *Brevibacillus parabrevis* SR2729. Glucose and $NaNO_3$ were observed as significant carbon and nitrogen sources, respectively. After RSM, the optimum activity of OPH was observed to be 365 U mL^{-1} at pH 4, temperature 52.5°C, incubation time 40.5h, carbon and nitrogen sources 0.5%, and pesticide concentration 6%. The results showed that at 6% of pesticide, low nutrients concentration, and short incubation time, the enzyme production was maximum, which could be a breakthrough for the degradation of pesticide. Such an optimized production of OPH can be practiced for the enhanced pesticide determination and degradation settings.

Key words: *Brevibacillus*, RSM, organophosphate, hydrolase, optimization.

Organophosphate pesticides are frequently used in Agriculture and Veterinary practice due to high efficiency. Applications on plants and soil cause their leeching and contamination to surface and ground water, which ultimately disturbs biological system (Kulkarni *et al*., 2000). In addition to its least solubility in water, and high stability in air, chlorpyrifos has been reported to be non-sensitive to ultraviolet radiation (Islam *et al*., 2010). Because of high absorption coefficient for soil while a less solubility in water, the possibilities for the contamination of water and terrestrial ecosystems by chlorpyrifos are higher as reported by EPA (Environmental Protection Agency) (Latifi *et al*., 2012). The enzyme organophosphate hydrolase is the active agent for the degradation of organophosphates in microbial cell factories. The enzyme degrades the insecticides by environmentally friendly method and converts them into less toxic products, while other methods of degradation by chemical agents fail the environment protection parameters. Increased applications of the enzyme OPH for the degradation of organophosphate compounds demands improved production by the process optimization. Vijayalakshmi and Usha, (2012) studied the effect of various factors (pH, temperature, carbon and nitrogen sources) for the improved degradation of chlorpyrifos.

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Traditional method for process optimization involves the experimentation of "one factor at a time". The approach fails to discriminate the combined effects of all the variables to give rise to optimized yield. In such procedures, the effects of interactions among the factors are also not taken into account (Zhang *et al*., 2010). In addition to these disadvantages, the classical approach is time consuming, laborious and requires large number of experiment to find out the optimum levels, which is highly unreliable. Such limitations of the classical approach can be eradicated by the statistical technique "response surface methodology" (RSM) (Ravikumar *et al.,* 2006). The process optimization is an important step for the enhanced production of the enzymes. Production of several enzymes has been improved by the process optimization. Sangyoung *etal*. (2004) also said that the classical/or conventional methods of studying the effect of a factor, while keeping all others at unspecified constant levels does not depict the combined effect of all the factors involved in the process. RSM is the combination of mathematical and statistical technique which is useful for process optimization of parameters for enhanced production of the enzymes. The technique improves the optimization process by evaluating the relative significance of several factors affecting the product yield. By the design of experiments, the series of experiments (runs), in which changes are made in the independent variables in order to identify the changes in the dependent variables (response/yield). Such RSM based designs are significantly used to reduce the cost of the expensive experimental process and the associated numerical noise. Here, we studied the effect of different carbon and nitrogen sources on the production of organophosphate hydrolase, while other factors (pH, temperature, incubation, time, carbon and nitrogen sources and pesticide concentrations) were studied by response surface methodology.

MATERIALS AND METHODS

Strain maintenance

The bacterial strain *Brevibacillus parabrevis* SR2729 (Accession no. KF952775) was previously isolated as a part of research work of Miss Samreen Rasul in Enzyme Biotechnology Laboratory. The strain was maintained in LB agar medium (NaCl 10, yeast extract 5.0, tryptone 10, pH 7.0 and temperature 30-35°C) unless used for further experimentation(Rani *et al.*, 2008).

Effect of carious carbon and nitrogen sourcecs on the production of OPH by parental and mutant derived strains

For the process optimization for enhanced production of OPH by parental and mutant derived strains, the preliminary tests for the effect of carbon and nitrogen sources were performed to obtain the best carbon and nitrogen source. In this regard, five carbon sources (glucose, sucrose, starch, maltose, lactose) and nitrogen sources (yeast extract, potassium nitrate, sodium nitrate, peptone and ammonium nitrate) were studied at the levels of 0.0, 0.02, 0.04, 0.06, 0.08 and 0.1 %. The enzyme extract obtained was analyzed for OPH activity by the method mentioned in section (Enzyme assay for OPH). The statistical analysis was performed by 2 factorial CRD design. The best carbon and nitrogen sources were finally utilized for the optimization of levels by Response Surface Methodology (RSM) (Vijayalakshmi and Usha, 2012).

Enzyme assay for OPH

The enzyme suspension was subjected to analysis by suspending its 0.1 mL in 0.9 mL CHES buffer (50 mM) pH 9.0, containing 0.2 mM chlorpyrifos as substrate, 10 % methanol, 2.5 % polyethylene glycol (PEG) 8000, 0.1% polyoxyethylene-10-laurylether. The reaction mixture was incubated at 37 °C for 5 minutes and noted the absorbance for hydrolysis of chlorpyrifos at 276 nm; where μ_{276}=2,790 $M^{-1}cm^{-1}$. The blank reaction mixture lacked the enzyme suspension 0.1 mL. The optical density was used to calculate the enzyme activity (Cho *et al*., 2004).

Optimization of conditions by Response Surface Methodology

The bacterial cells (parental and mutant derived) subjected to process optimization for optimized production of OPH. Response surface methodology was employed for the analysis of optimization medium. Six parameters (pH, temperature, incubation time, carbon source, nitrogen source, and pesticide concentration %) were selected for optimization. The levels and design for RSM is shown as under (Table 1). A quadratic polynomial desigh was prepared for

the process optimization of OPH production using parental and mutant derived strains of new bacterial isolate. The design expert 9 was used to formulate the response surface design for the process optimization of OPH. Six factors with three models were tested in 54 runs. The factors were pH, temperature, incubation time (h), carbon source %, nitrogen source %, and pesticide concentration %. All the six factors were coded as A, B, C, D, E, and F, respectively. All the experiments were run in triplicates to minimize the errors.

RESULTS AND DISCUSSION

Effect of carbon sources on OPH production

The enzyme production is greatly affected by the addition of different carbon sources at variable concentrations. The carbon sources cause the change in the yield of the end product by affecting the rate at which the carbohydrates are metabolized (Abdullah *et al.,* 2003). The highest production of OPH by parental mutant derived strains was observed when glucose was used as carbon source, while less production of enzyme was observed by using sucrose, starch, maltose and lactose (Fig. 1). The level of glucose for the production of OPH was 0.08%, while the lower activity of OPH was observed for other carbon sources. Two factor CRD design was performed for the determination of statistical significance of the obtained data.

ANOVA for the process optimization of carbon sources is shown in Table 2. The results for sources, levels and source × level interaction affect were highly significant for the carbon sources for the production of OPH.Glucose showed the significant source × level interaction mean ± standard error, with "A" value of 209.53±9.07 at 0.06% glucose concentration. The interactioneffect of glucose with levels is indicated by small "a" with a value of 283.00±1.53 at 0.08 % glucose concentration. While, the main and interaction effects of all other carbon sources remain non-significant for OPH production from *B. parabrevis* SR2729.On the basis of results, glucose was selected as best suited carbon source for the optimized production of OPH. The results are in accordance with Vijayalakshmi and Usha, (2011), where they observed highest degradation of chlorpyrifos by using glucose as carbon source. However, their reported strain *Pseudomonas putida* utilized a high concentration of glucose for the optimized degradation of chlorpyrifos. Tsai *et al.* (2012) reported 0.1 % glucose as carbon source for the production of OPH enzyme by *Enterobacteraerogenes* strain. Ghribi and

Table 1. Intermediate, Low and high levels of all six factors for RSM design

Factor	Name	Intermediate level	Low level	High level
A	pH	7.00	4.00	10.00
B	Temperature °C	52.50	25.00	80.00
C	Incubation time (h)	40.50	9.00	72.00
D	C-source %	0.050	0.000	0.100
E	N-source %	0.050	0.000	0.100
F	Pesticide %	6.00	2.00	10.00

Table 2. Analysis of variance table for optimization of carbon sources for OPH production by strain *B. parabrevis* SR2729

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F-value
Source	4	67818	16954.5	155.28**
Level	5	102879	20575.8	188.45**
Source x Level	20	47739	2387.0	21.86**
Error	60	6551	109.2	
Total	89	224988		

** = Highly significant (P<0.01)

Chaabouni, (2011) reported the optimum production of lipopeptidebio surfactant by *Bacillus subtilis* by using glucose (40 gL^{-1}) as carbon source. It is reported that effect of carbon source changes with the production strain and other production conditions. Maximum production of amylase was obtained after using starch as carbon source in production medium for *Bacillus sp. marini*. (Ashwini *et al*., 2011).

Table 3. Analysis of variance table for optimization of nitrogen sources for OPH production by strain *B. parabrevis*

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F-value
Source	4	27252.4	6813.09	694.43**
Level	5	30409.2	6081.83	619.89**
Source x	20	11179.0	558.95	56.97**
Level	60	588.7	9.81	
Error Total	89	69429.2		

** = Highly significant (P<0.01)

Table 4. Analysis of variance table for the optimization of OPH by *B. parabrevis* SR2729

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F	
Model	7.502E+005	27	27784.97	84.59	< 0.0001	Significant
A-pH	76050.04	1	76050.04	231.52	< 0.0001	Significant
B-temp.	36973.50	1	36973.50	112.56	< 0.0001	Significant
C-incubation	59302.04	1	59302.04	180.53	< 0.0001	Significant
D-C-source	28842.67	1	28842.67	87.81	< 0.0001	Significant
E-N-source	3037.50	1	3037.50	9.25	0.0053	Significant
F-Pesticide	39204.17	1	39204.17	119.35	< 0.0001	Significant
AB	10731.13	1	10731.13	32.67	< 0.0001	Significant
AC	6105.13	1	6105.13	18.59	0.0002	Significant
AD	18632.25	1	18632.25	56.72	< 0.0001	Significant
AE	7503.13	1	7503.13	22.84	< 0.0001	Significant
AF	5050.13	1	5050.13	15.37	0.0006	Significant
BC	11552.00	1	11552.00	35.17	< 0.0001	Significant
BD	325.13	1	325.13	0.99	0.3290	non-significant
BE	473.06	1	473.06	1.44	0.2409	non-significant
BF	3741.13	1	3741.13	11.39	0.0023	Significant
CD	10658.00	1	10658.00	32.45	< 0.0001	Significant
CE	162.00	1	162.00	0.49	0.4888	non-significant
CF	16192.56	1	16192.56	49.30	< 0.0001	Significant
DE	190.13	1	190.13	0.58	0.4536	non-significant
DF	3698.00	1	3698.00	11.26	0.0024	Significant
EF	1.13	1	1.13	3.425E-003	0.9538	Significant
A^2	15026.79	1	15026.79	45.75	< 0.0001	Significant
B^2	2.407E+005	1	2.407E+005	732.74	< 0.0001	Significant
C^2	81422.29	1	81422.29	247.87	< 0.0001	Significant
D^2	26593.10	1	26593.10	80.96	< 0.0001	Significant
E^2	4742.29	1	4742.29	14.44	0.0008	Significant
F^2	1.015E+005	1	1.015E+005	309.05	< 0.0001	Significant
Residual	8540.54	26	328.48			
Lack of Fit	6999.21	21	333.30	1.08	0.5155	not significant
Pure Error	1541.33	5	308.27			
Cor. Total	7.587E+005	53				

Effect of nitrogen sources

Nitrogen sources affect the microbial cell factories for the production of important metabolites. In present research work, five nitrogen sources yeast extract, KNO_3, $NaNO_3$, Peptone and NH_4NO_3 were utilized for the optimization of OPH production. Where yeast extract and peptone were organic nitrogen sources, while other three were inorganic sources. By observing the activity of OPH at variable levels of these nitrogen sources, it was found that the *B. parabrevis* SR2729 utilized $NaNO_3$ as best suited nitrogen source to produce OPH (Fig. 2). After the statistical analysis of the data obtained by the parental and mutant derived strains the ANOVA for the all three strains showed the significance of source, level, and source × level interaction of nitrogen sources for OPH production. Table 3 shows Source x level interaction mean±SE for nitrogen sources optimization for OPH production by the *B. parabrevis* SR2729. The table shows the significance of $NaNO_3$ as main factor for the production of OPH, with mean × SE of 147.72 ±7.71. The interaction effect of $NaNO_3$ × levels was also significant with mean × SE 139.87 ±6.62 at the level of 0.06%. The results were not in correlation with Vijayalakshmi and Usha, (2011). They tested a range of nitrogen sources for the optimization of conditions for degradation of chlorpyrifos. They observed that in the presence of yeast extract as nitrogen source, the highest degradation of chlorpyrifos was obtained, while peptone, $NaNO_3$, $NH4NO_3$ and KNO_3 followed yeast extract. Process optimization for chlorpyrifos degradation or production of OPH enzyme has been a limited area of research in previous literature. Additionally, much attention is required on the better and optimized OPH production for the bioremediation of chlorpyrifos from the environment.

Process optimization of OPH production by RSM

By the examination of six factors interaction at variable levels, 54 experimental runs were performed. The second order polynomial coefficient for each term of equation "x" were determined through multiple regression analysis using Design expert 9. Chen *et al*. (2012) used CCRD for the optimization of degradation of

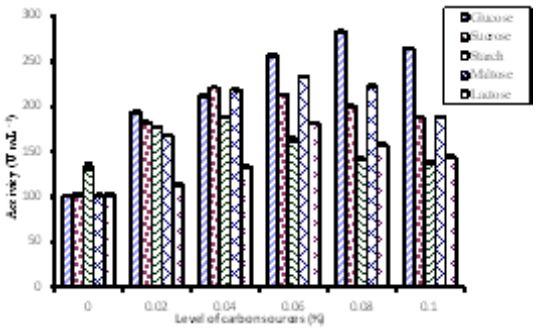

Fig. 1. Effect of carbon sources on the production of OPH by strain *B. parabrevis* SR2729

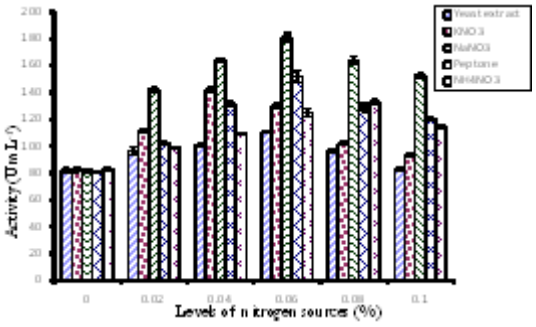

Fig. 2. Effect of nitrogen sources on the production of OPH by strain *B. parabrevis* SR2729

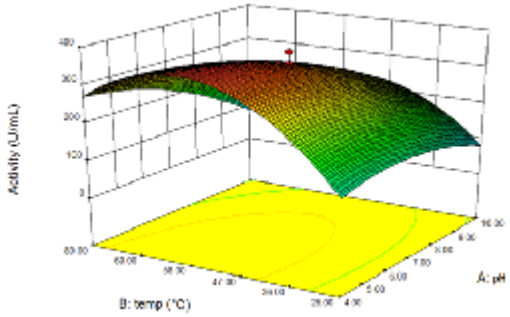

Fig. 3. Statistical 3D surface model of the combined effect of pH and temperature for OPH activity from strain *B. parabrevis* SR2729

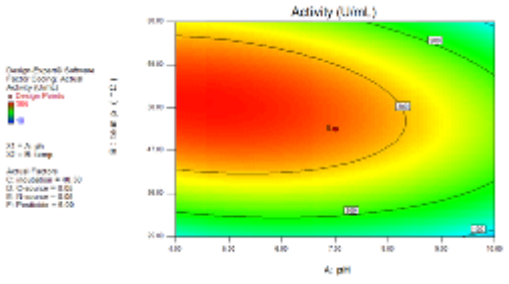

Fig. 4. Contour plot for interaction of pH and temperature for process optimization of OPH by *B. parabrevis* SR2729

chlorpyrifos with three factors at five variable points. The three factors were pH, temperature, and culture time for the chlorpyrifos degradation.

Regression Equation

U/mL activity of OPH (y) = (+331.67) + (-56.29A) + (+39.25B) + (+49.71C) + (+34.67D) + (+11.25E) + (+40.42F) + (-36.63AB) + (-27.63AC) + (-34.12AD) + (+30.63AE) + (-25.12AF) + (+38.00BC) + (+21.62BF) + (+31.81CF) + (+21.50DF) + (+0.37EF) + (-38.22A^2) + (-152.97B^2) + (-88.97C^2) + (-50.85D^2) + (-21.47E^2) + (-99.35F^2)…… "x"

ANOVA is a statistical technique that subdivides the total variation in a set of data into component parts associated with specific sources of variation for the purpose of testing hypothesis on the parameters of the model. According to ANOVA the F-value of 84.59 implies that the model is significant and there are only 0.01% chances that a larger F-value of Model could be due to noise. Model terms were tested at 5% confidence level, which is P>F value and less than 0.05 indicate the significance of model terms (Table 4). Here, model terms pH, temperature, incubation time, carbon source, nitrogen source, and pesticide concentration are significant. In addition the pH-temperature, pH-incubation time, pH-carbon source, pH-nitrogen source, pH-pesticide concentration, temperature-incubation time, temperature-pesticide concentration, incubation time-carbon source, carbon source-pesticide concentration were also significant. The quadratic effects of pH, temperature, incubation time, carbon source, nitrogen source, and pesticide concentration were also significant. On the other hand values greater than 0.1000 indicate that the model terms are non-significant. Here, temperature-carbon source, temperature-nitrogen source, incubation-nitrogen source, and carbon source-nitrogen source are non-significant terms with Prob>F values of 0.3290, 0.2409, 0.4888, and 0.4536, respectively.

Lack of fit test for the present model obtained the 1.08, which implies that the model is lack of fit is non-significant, relative to pure error. There are 51.55% chances that, this lack of fit could be due to noise. Non-significant lack of fit term indicates that fit model. The ANOVA table also presented the value of residual error, which measures the amount of variation in the response data left unexplained by the model. The form of model chosen to explain the correlation between the factors is correct. Predicted R-squared (0.9489) was in reasonable agreement with adjusted R-squared (0.9771), the difference was less than 0.2. The regression R^2 was also found to be 0.988, indicating that 98.8% variability in the model could be explained by the model. It also implies that the precision of the model and experimental data is quite satisfactory.

Response surface plots, as a function of two factors at a time, where all other four factors maintained at fixed level, are suitable to understand both the main and interaction effects of the factors. The values of the responses were used to formulate the response surface curves to determine the optimum levels of the factors for maximum response. The response surface curve for the obtained activity of OPH from strain *B. parabrevis* SR2729is presented in Fig. 3. The curve presented the interaction between six variable factors; where the significant interaction between variable was presented by the elliptical shape of the curve. Elavarasan *et al*, (2009) said that the elliptical shape of the curve signifies the good interaction between the process variables, while the circular shapes are indication of non-significant interaction among variables. Contour plot for the interaction between two variable factors for change in enzyme production is shown in Fig. 4.

The central/stationary point is the spot, where the slope of the contour is zero in all directions. The coordinated of the central spot, within highest contour levels in each of these figures corresponds to the optimum values of the respective components. Gopal *et al*. (2002) obtained the maximum color removal of the dye in the smallest curve of the contour diagram. The optimum values obtained from the images are in close agreement with the regression equation model "x". The optimum point was observed at the center of the contour diagram. Based on the graphical results as presented in Figure 3, the optimized production of OPH was obtained at pH 6; temperature = 52.5 °C; incubation time = 40.5 h; carbon and nitrogen sources = 0.5 and pesticide = 6%. The optimum response of 365 UmL^{1} was obtained after using the optimized values of all six factors. The application of RSM instead of classical approach of one factor at a time significantly reduced the empirical efforts required. The

possibilities, of determining the true optimal conditions for the production of OPH increased due to study on interaction effects of six variables at the same time.

CONCLUSION

The optimized medium for organophosphate hydrolase production contained the low levels of carbon and nitrogen sources, and less time of incubation. Additionally, low pH indicated the ability of bacterial strain for enhanced OPH production in acidic medium. Such a medium will be economically suitable for enhanced production of the enzyme. It was thus concluded that, the medium will be a good combination for the optimum production of organophosphate hydrolase (Activity = 365 U mL^{-1}).

ACKNOWLEDGEMENTS

The research work was performed at Enzyme Biotechnology Laboratory, Department of Biochemistry, University of Agriculture, Faisalabad. It was funded by Higher Education commission of Pakistan under the grant no. 117-5181-BM7-103 (50018229). Authors are grateful to Dr. AmerJamil and Dr. BushraSadia for laboratory facilities.

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