

Original Research Article

<https://doi.org/10.20546/ijcmas.2017.603.012>

Production of α -L-Rhamnosidase from *Aspergillus flavus*: Optimization of Submerged Culture Conditions by Taguchi DOE Methodology

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ABSTRACT

Keywords

α -L-rhamnosidase,
DOE (Design of
Experiment),
Bitterness, Citrus
fruit juice, Naringin.

Article Info

Accepted:
08 February 2017
Available Online:
10 March 2017

The enzyme α -L-rhamnosidase [E.C.3.2.1.40] specifically cleaves terminal α -L-rhamnose from a large number of natural products containing glycosides. This property provides this enzyme an important biotechnological application including removal of bitterness from citrus fruit juices, enhancement of grape wine aroma, removal of hesperidin crystals from orange juices, tomato pulp digestion and also help in conversion of clinical important steroids. In this study, production of α -L-rhamnosidase in submerged state fermentation from *Aspergillus flavus* was optimized using Taguchi DOE methodology. This statistical method provide the study of interaction of a large number of factors at different levels settings with a small number of experimental runs which leads to considerable economy in time and cost for the process optimization. The objective of this research was to determine the significant parameters for the production of α -L-rhamnosidase by *Aspergillus flavus* in submerged fermentation. Five factors Viz. inducer concentration (naringin), incubation days, temperature, pH and metal ion at two levels were selected with orthogonal array layout of L16 (2^5). The experimental result indicate that inducer concentration (0.5% naringin), incubation days (7), temperature (28 °C), and pH (6.0) were the important factors for α -L-rhamnosidase production by *Aspergillus flavus* in submerged state fermentation at the optimum levels.

Introduction

The biotechnological potential of microbial enzymes has drawn a great deal of attention from various researchers worldwide, as they are likely to be biological catalysts in a variety of industrial processes, high production yields, easy genetic manipulation, provide regular production due to independent of geographical condition, fast growth of microorganism in inexpensive media, more convenient and safer protection methods. Among microbial enzymes, α -L-rhamnosidase [E.C.3.2.1.40] which cleaves

terminal α -L-rhamnose from a large number of natural glycosides includes mainly naringin, rutin, hesperidin quercitrin and terpenyl glycosides (Yadav *et al.*, 2010). This enzyme is a part of an enzyme complex called naringinase. Naringinase has two subunits: α -L-rhamnosidase which hydrolyzenaringin to rhamnose and prunin, which are further converted into glucose and naringenin by another subunit β -D-glucosidase [EC.3.2.1.21] (Puri *et al.*, 2005). This enzyme has been reported in literature from various

sources such as plants, animal tissue, yeast, fungi and bacteria.

α -L-rhamnosidase has several applications in food industry mainly removal of bitter compound naringin from citrus fruit juice by converting to less bitter compound prunin (Puri *et al.*, 2010) and elimination of hesperidin crystals from citrus juices (Miyake *et al.*, 1999). In wine industry, α -L-rhamnosidase play important role in aroma enhancement of grape fruit wine (Manzanares *et al.*, 2000), in pharmaceuticals industry it's used for derhamnosylation of natural compound containing the L-rhamnose residues in the terminal and biotransformation of steroids and antibiotics (Thirkettle, 2000).

Among fungi, *Aspergillus flavus* is very prominent producer of α -L-rhamnosidase. It is well known that α -L-rhamnosidase is an extracellular enzyme which production in submerged fermentation is greatly affected by inducer concentration, incubation days, pH, temperature and metal ions. Conventional optimization procedures involve changing of one factor at a time keeping all other parameters constant. These processes are very time consuming, complicated, and require more experimental data sets and also can't provide more information about interaction of the factors (Beg *et al.*, 2003). 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.

Alternative to conventional optimization procedures, Taguchi Design of experiments (DOE) provide more information about the optimization process in a few trials (Krishna *et al.*, 2005). Statistical experimental design methods provide an efficient and systematic plan for optimization of culture conditions by considering the interactive effects among the

control factors. Several control factors can be simultaneously analysed and optimized by statistical experimental design tool in Taguchi DOE (Rao *et al.*, 2004; Abdel *et al.*, 2005).

In this study, Taguchi Design of Experiment (Minitab 17) was used to optimize the submerged culture conditions for the production of α -L-rhamnosidase. The experiments were designed for five factors inducer concentration, incubation days, temperature, pH and metal ion at two levels with orthogonal array layout of L16.

Materials and Methods

Chemicals

Naringin and *p*-nitrophenyl- α -L-rhamnopyranoside were obtained from Sigma, India. The other culture media (Potato dextrose Agar, Czapek Dox Broth) and different carbon and nitrogen sources were obtained from Hi-Media. All other reagents were used of analytical grade.

Microorganism and culture media

The fungal strain used in present study, *Aspergillus flavus* was screened and isolated from decaying lemon peel and culture was maintained at 4⁰C in Potato Dextrose Agar slants (PDA) throughout the experiments and sub-cultured in every two weeks.

Production of α -L-rhamnosidase

The production of α -L-rhamnosidase enzyme was done in the submerged culture condition in which Czapek Dox Broth was used. 100 ml of culture media in 250 ml Erlenmeyer flask was inoculated with 5 mm bead size of 48 hours grown isolated fungal strain from PDA plate. Different concentrations of inducer (naringin) range between 0.3 and 0.5% were used for enzyme production. The flask was

incubated in B.O.D incubator at 28⁰C in stationary conditions. The crude enzyme was obtained by centrifugation of the culture broth at 10000 rpm for 10 minutes at 4⁰C. The supernatant which contains the enzymes was assayed for α -L-rhamnosidase activity.

Enzyme assay

The activity of α -L-rhamnosidase was assayed by Davis method (1947) in which naringin was used as a substrate. The reaction solution constituted of 1.0 mL of 5% naringin dissolved in 0.1 M sodium acetate buffer, pH 4.0, maintained at 50 °C for 1 hour. 0.2 mL of enzyme extract was added to the above solution. From the incubated mixture, 0.1 ml was added to 5 ml of 90% diethylene glycol containing 0.1 ml 4 M NaOH. After 10 minutes at room temperature, the residual naringin, the intensity of the resultant yellow colour, was measured at 420 nm. One unit of α -L-rhamnosidase activity (U) is defined as the amount of enzyme that could hydrolyze one micromole of naringin per minute at the assay conditions. Another method was also used to check the activity of α -L-rhamnosidase enzyme by using synthetic substrate *p*-nitrophenyl- α -l-rhamnopyranoside and monitored the liberation of *p*-nitrophenolate ion spectrophotometrically at 400 nm, molar extinction coefficient value of 21.44 mM⁻¹ cm⁻¹ (Romero *et al.*, 1985).

Optimization methodology

Minitab 17 Taguchi software (e-academy) was used to optimize the α -L-rhamnosidase production with the help of Taguchi Design of Experiment. In this experimental design a standard orthogonal array L16 (2⁵) with 15 degree of freedom was used to examine five factors at two 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 analysed to find out the effects of main factors; the analysis of variance (ANOVA) was then applied to know which factors were statistically significant. The controlling factors were identified, and then optimized statistically significant variables. Also the optimum conditions were determined by combining the different levels of factors that showed the highest main effect value.

Results and Discussion

Taguchi experimental design is a good statistical tool for the optimization of biotechnological processes for production of microbial enzymes. It is the sequence of steps which initially identify the factors to be optimized and then test condition to ensure that the data obtained in this analysis will lead to valid statistical interpretation.

In this study, the influence of 5 factors on the α -L-rhamnosidase production by *Aspergillus flavus* was tested in Taguchi experimental design in 16 runs by using Minitab 17 software (e-academy).

The results of Taguchi experimental design in 16 runs, for the five factors, inducer concentration (naringin), incubation days, temperature, pH and metal ion at two level selected for optimization of α -L-rhamnosidase production by the fungal strain *Aspergillus flavus* (Table 2) show the efficiency of α -L-rhamnosidase production ranging from 0.40 – 3.88 U / mL corresponding to the combined effect of the five factors in their specific ranges. The experimental results justified that these factors at optimized level support the production of α -L-rhamnosidase.

In run 2, inducer concentration (0.3 % naringin), incubation days (4), temperature (25⁰C), pH (6) and metal ion (0.07%), showed lowest production 0.40 U/ml was observed. The production of 3.88 U/ml was observed in run 16 with a combination of inducer concentration (0.5 % naringin), incubation days (7), temperature (28⁰C), pH (6) and metal ion (0.07%). Figure 1 represents the contribution of selected factors for the α -L-rhamnosidase production in submerged state fermentation. It can be observed that inducer (naringin), incubation days, pH and temperature respectively contributing of 25.35 %, 21.11 %, 18.56 % and 18.32 % have shown highest positive impact on the α -L-rhamnosidase enzyme 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 α -L-rhamnosidase production was carried out according to the factors which contributed more than 10% as suggested by the Taguchi method.

In Taguchi Design of Experiments, ANOVA was used to analyze the results of the Orthogonal Array experiments and determine how much variation of each factor has contributed. By studying the main effects of each and every factors, the general trends of the influence of the factors towards the process can be categorized. The characteristics can be controlled such that a lower or a higher value in a particular influencing factor produces the significant result. Thus, the levels of factors to give the best results can be predicted (Krishna *et al.*, 2005).

Analysis of the data for the determination of significant parameters on α -L-rhamnosidase production and the results are shown in ANOVA table 3. From the calculated ratios (F), it can be referred that the factors

considered in the Taguchi experimental design are statistically significant at 95% confidence limit. The ANOVA of α -L-rhamnosidase production has the model F-value of 137.94 that implies the model is significant. The model obtained from ANOVA indicated that the multiple correlation coefficient of R² is 0.9857 i.e. the model can explain 98.57% variation in the response. Also, the model has an adequate precision value of 9.371; this suggests that the model can be used to navigate the design space. The adequate precision value is define as an index of the signal to noise ratio and the value of signal to noise ratio should be greater than 4 then the model is good fit. The model shows standard deviation, mean, coefficient of variance and predicted residual sum of square (PRESS) values of 6.20, 121.57, 4.47 and 324.79 respectively. Point prediction for achieving highest α -L-rhamnosidase enzyme production in terms of levels of factors is shown in table 4. Under optimal conditions for α -L-rhamnosidase, the expected activity was 3.88 U/mL.

Figures 2 represent the main effect plots for the means which show how each factor affects the response characteristic. A main effect is present when different levels of a factor affect the characteristic differently. MINITAB 17 creates the main effects plot by plotting the characteristic average for each factor level. A line connects the points for each factor. When the line is parallel to the x-axis, i.e. horizontal, it showed there is no main effect present.. When the line is not parallel to the x-axis, it shows that there is a main effect present. Different levels of the factor affect the characteristic differently. The greater the difference in the vertical position of the plotted points i.e. to parallel x - axis, the greater is the magnitude of the main effect was shown in the figure 2.

Table.1 Factors and their levels employed in the Taguchi experimental design method for optimization of α -L-rhamnosidase production from *Aspergillus flavus* in submerged state fermentation

Factors	Level 1	Level 2
Inducer (Naringin)	0.3%	0.5%
Incubation Days	4	7
Temperature ($^{\circ}$ C)	25	28
pH	5	6
Metal Ion (0.07%)	Ca ⁺⁺	Cu ⁺⁺

Table.2 L16 (25) orthogonal array of Taguchi design of experiments and corresponding α -L-rhamnosidase production from *Aspergillus flavus* in submerged state fermentation

Run	Inducer (% of Naringin)	Incubation Days	Temperature ($^{\circ}$ C)	pH	Metal Ions (0.07%)	Response Enzyme Activity (U/ml)
1.	0.3	4	25	5	Ca ⁺⁺	0.48
2.	0.3	4	25	6	Cu ⁺⁺	0.40
3.	0.3	4	28	5	Cu ⁺⁺	0.62
4.	0.3	4	28	6	Ca ⁺⁺	0.68
5.	0.3	7	25	5	Cu ⁺⁺	1.13
6.	0.3	7	25	6	Ca ⁺⁺	1.91
7.	0.3	7	28	5	Ca ⁺⁺	1.82
8.	0.3	7	28	6	Cu ⁺⁺	1.72
9.	0.5	4	25	5	Cu ⁺⁺	2.28
10.	0.5	4	25	6	Ca ⁺⁺	2.80
11.	0.5	4	28	5	Ca ⁺⁺	2.68
12.	0.5	4	28	6	Cu ⁺⁺	2.88
13.	0.5	7	25	5	Ca ⁺⁺	3.38
14.	0.5	7	25	6	Cu ⁺⁺	3.11
15.	0.5	7	28	5	Cu ⁺⁺	3.32
16.	0.5	7	28	6	Ca ⁺⁺	3.88

Table.3 ANOVA for α -L-rhamnosidase production by *Aspergillus flavus*

Analysis of Variance (ANOVA)					
Source	DF	Sum of Square	Mean Square	F-Value	P-Value
Model	10	19.1604	3.8321	137.94	0.002*
Inducer	2	15.0156	15.0156	540.52	0.003*
Incubation Days	2	3.4040	3.4040	122.54	0.001*
Temperature	2	0.2601	0.2601	9.36	0.012*
pH	2	0.3906	0.3906	14.06	0.004*
Metal Ion.	2	0.0900	0.0900	3.24	0.102
Residual	5	0.2778	0.0278		
Total	15	19.4382			

Abbreviation: * Significant

Table.4 Point prediction for optimum conditions of α -L-rhamnosidase production from *Aspergillus flavus* in submerged state fermentation

	Prediction	SE Mean	95 % CI Low	95 % CI High	Optimum conditions
α -L-rhamnosidase activity (U/ml)	2.99327	0.850891	109.785	188.965	Inducer (Naringin 0.5%), Incubation days (7), Temperature (28 °C), pH (6), Ca ⁺⁺ (0.07%)

Abbreviation: SE: Standard Error, CI: Confidence Interval

Figure.1 Contribution of five factors on α -L-rhamnosidase production from *Aspergillus flavus* in a submerged culture using Taguchi experimental design

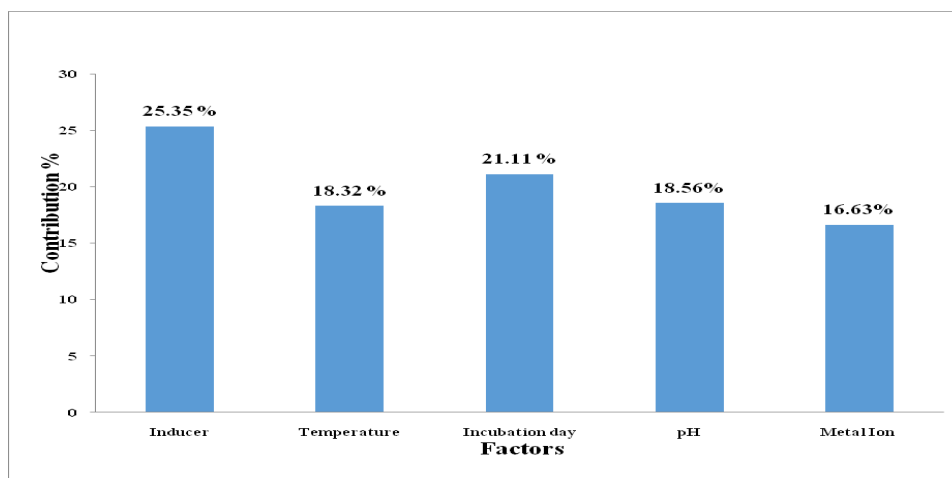
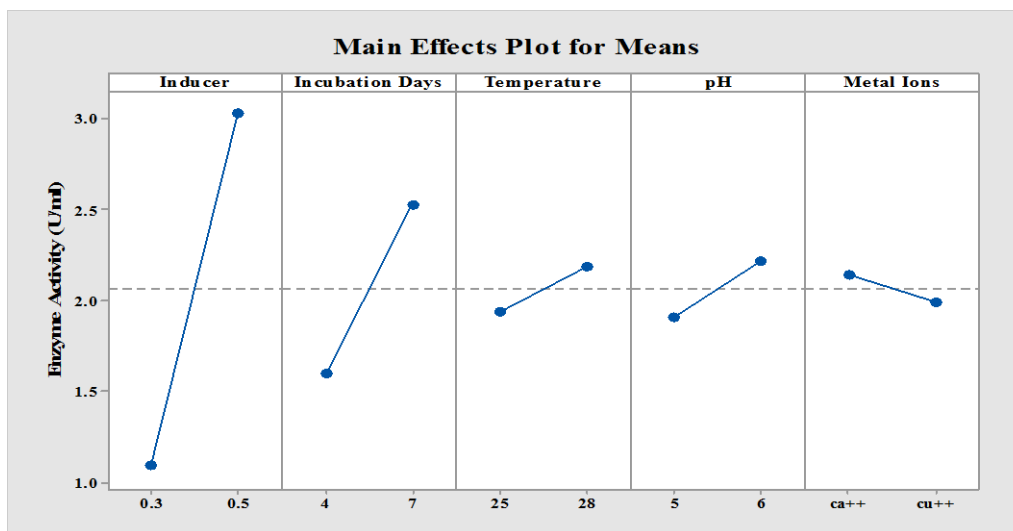


Figure.2 Graph showing the effect of each factor contribution in the α -L-rhamnosidase enzyme production by *Aspergillus flavus* in submerged state fermentation



Each microorganism has its own special conditions for maximum enzyme production. The isolated fungus *Aspergillus flavus* from decaying citrus peel showed the highest enzyme activity when media composition is optimized. Although fungi of the genera *Penicillium* have been also reported as producers of this enzyme (Elinbaum *et al.*, 2002). *Aspergillus* genus had been used preferentially for α -L-rhamnosidase enzyme production for a number of years. This is because of its high level of extracellular enzyme production seen in this genus. In this study, Taguchi Design of Experiment for α -L-rhamnosidase production by *Aspergillus flavus* and reported this experiment design provided basic information to improve the efficiency of α -L-rhamnosidase production and also supported the analysis of the main factor of each constituent of the medium. Adriana *et al.*, (2014) also successfully applied Taguchi experimental design for α -L-rhamnosidase production by *Aspergillus niger* and observed enhanced production.

Among various statistical designs of experimental methods, Taguchi DOE is the most reliable statistical method and offers enhanced yields in just few experimental

trials that provide the interactive effects among the various process factors and their levels. It's reduces both the time required for achieving optimum activity of enzyme and as well as cost of production. It is reported that statistical DOE have been employed for increasing production yields in different studies (Kunamneni *et al.*, 2005). Any particular factor may interact with each other's and then provide the possibility of presence of interactions among them. This type of interaction is possible in Taguchi design of experiment. The estimated interaction severity index (SI) of the factors understudy helps to know about the influence of two individual factors at various levels of the interactions (Koo *et al.*, 2006).

In this study, α -L-rhamnosidase production by *Aspergillus flavus* in submerged state fermentation, inducer concentration (0.5 % naringin), incubation days (7), temperature (28 °C), pH (6) and metal ion (0.07%) up to level 2, constituted the main factors of the medium. The contribution of five factors in α -L-rhamnosidase production by Taguchi experimental design showed that inducer concentration (naringin) played a leading role than the other selected parameters (inducer

(naringin) 25.35 %, incubation days 21.11 %, pH 18.56 % and temperature 18.32 % and metal ions 16.63 %). Point prediction of the design showed that maximum α -L-rhamnosidase production of 2.99 U/ mL was achieved under optimal experimental conditions. This result would further provide economic design of the large scale fermentation operation system for biotechnology processes.

In conclusion, Taguchi Design of Experiment (DOE) by using Minitab 17 was successfully applied in this study to test the relative importance of medium components and factors on α -L-rhamnosidase production. The selected orthogonal array was L16 (2^5) and optimum factors for α -L-rhamnosidase production were found to be inducer (naringin), incubation days, temperature and pH. The optimum medium condition derived was: inducer concentration (0.5% naringin), incubation days (7), temperature (28°C), and pH (6). At this optimum condition, the yield of α -L-rhamnosidase production by *Aspergillus flavus* was found to be 3.88 U/mL.

Acknowledgement

One of the author, Poonam Yadav want to thanks the Department of Science and Technology (DST), Government of India, for awarding DST-INSPIRE fellowship for financial support to carry out this work at Centre of Food Science and Technology, Banaras Hindu University, Varanasi.

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How to cite this article:

Poonam Yadav, Anil Kumar Chauhan, Mohammed A. Al-Sebaei and Suman Yadav. 2017. Production of α -L-Rhamnosidase from *Aspergillus flavus*: Optimization of Submerged Culture Conditions by Taguchi DOE Methodology. *Int.J.Curr.Microbiol.App.Sci.* 6(3): 110-118. doi: <https://doi.org/10.20546/ijcmas.2017.603.012>