

Enhanced Production of Acyltransferase from *Rhodococcus pyridinivorans* using Statistical Experimental Design

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Abstract: Optimization of growth parameters for enhanced production of acyltransferase by *Rhodococcus pyridinivorans* were carried out using one-variable-at-a-time strategy (OVAT) and statistical designs of response surface methodology (RSM) to describe the relationship between tested variables. Optimization resulted into 3.8 folds increase in the acyltransferase production ($448.44 \pm 7.00 \text{ U mg}^{-1} \text{ dcw}$). Based on results of Plackett-Burman design, combined effect of variables viz. pH, temperature, inoculum size and non-substrate inducer acetonitrile on production of acyltransferase were investigated at four levels, through the statistical analysis of central composite design (CCD). The statistical optimization showed further increase in acyltransferase activity of 1.06 folds. The validation of the experiments was also done by point determination in generated model with actual and predicted response level under optimized condition which resulted acyltransferase production of $480.8 \pm 2.01 \text{ U mg}^{-1} \text{ dcw}$ (Predicted response $474.71 \text{ U mg}^{-1} \text{ dcw}$). After performing optimization of all production parameters, 4.2 fold increase in acyltransferase production ($519.03 \pm 1.093 \text{ U mg}^{-1} \text{ dcw}$) was recorded with 3.0 % (w/v) acetamide, 1.5 % (w/v) yeast extract, 0.5 % (w/v) NaCl, 0.2 % (w/v) glucose, pH 7.0 at 30°C, 8 % (v/v) inoculum size and acetonitrile in multistep feeding as non substrate inducer (70 mM) for 18 h.

Keywords: *Rhodococcus pyridinivorans*, one variable at a time (OVAT), central composite design (CCD), acyltransferase production.

Introduction

Amidases or amidohydrolase is the nitrilase super family enzymes which are prevalent in prokaryotes and eukaryotes. The biological functions of amidases vary widely, but they are typically involved in carbon and nitrogen metabolism in prokaryotes. In nitrile metabolism, amidases possess dual catalytic activity viz. hydrolysis of amides to corresponding acids and acyltransferase activity in presence of hydroxylamine to form hydroxamic acids²². The use of acyl transfer activity of amidase may be used to convert amides to hydroxamic acids. In acyltransferase catalyzed biotransformation, amides acts as acyl-group donors and hydroxylamine as acyl-group acceptors.

Acyltransferase activity of amidase is exploited mainly for the synthesis of pharmaceutically active hydroxamic acids^{29, 14, 11}. Hydroxamic acids are known to possess high chelating properties. Some of hydroxamic acids such as α -aminohydroxamic acids, synthetic siderophores, and acetohydroxamic acid have also been investigated as anti-human immunodeficiency virus agents or anti-malarial agents or have been recommended for treatment of urea plasma infections and anaemia^{9, 14}. Moreover, some fatty hydroxamic acids have been studied as inhibitors of cyclooxygenase and 5-lipoxygenase with potent anti-inflammatory activity¹².

Amidases from microorganisms have gained im-

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portance as industrial biocatalysts like synthesis of acrylamide and acrylic acid catalyzed by amidase is one of the largest industrial biotransformations in the world³⁶. Moreover, a wide range of commercially useful chemicals can be synthesized using microbial amidases³⁹. Microbial amidases are found abundantly in *Rhodococci*, *No-cardia*, and *Arthrobacteria*. *Rhodococcal* amidases exhibit fairly wide substrate specificity and often demonstrate stereoselective hydrolysis of amides of α -substituted carboxylic acids²⁵. Along with nitrile hydratases, amidases can catalyze the unique conversion of nitriles to the corresponding enantiopure carboxylic acids. *Rhodococcal* amidase and nitrile hydratase find applications in the stereoselective synthesis of several drugs and drug intermediates^{3, 36}. Hence, large scale fermentative production of *Rhodococcal* amidase is of great commercial interest. In order to make this process commercially viable, it is necessary to improve the yield without increasing the cost of production. One method of achieving this objective is selection of appropriate media components and optimal culture conditions. Thus, optimization of fermentation conditions is generally regarded as the most crucial primary step in the development of a cost-effective fermentation process²⁰. In present study, research efforts were focused on optimization of production medium with maximum yield of acyltransferase activity of amidase from *Rhodococcus pyridinivorans* by using traditional method one variable at a time and response surface methodology. Single variable optimization method is simple, time consuming and fails to locate the region of optimum response because the joint effects of factors on response are not taken into account in such procedures. To eliminate these limitation response surface methodology were also used which not only deals with experimental strategies but also deals with mathematical methods and statistical inference for constructing and exploring an approximate functional relationship between a response variable and a set of design variable^{27, 33}.

Chemicals

All the chemicals were of analytical grade. The nitriles and amides were purchased from Alfa

Aesar, A Johnson Matthey Company (earlier Lancaster Synthesis). Media components were purchased from Hi Media (Mumbai) and the inorganic salts were of analytical grades.

Materials and Methods

Microorganism

In the present study, a nitrile and amide degrading bacterium (*Rhodococcus pyridinivorans*) was earlier isolated from soil sample of Shimla (Himachal Pradesh, INDIA). This bacterium has been identified by 16S rRNA genes sequencing analysis and the sequence has been deposited in NCBI GenBank database with accession number KU837232. It been explored for its acyltransferase (amidase) activity. Since the acyl transfer activity has been studied so the protein is being addressed as acyltransferase, though it is an amidase.

Preparation of resting cells of *Rhodococcus pyridinivorans*

A loopful of bacterial cells (*Rhodococcus pyridinivorans*) from the slant were seeded in 50 ml of modified nutrient broth containing 5 g peptone; 3 g beef extract; 1 g yeast extract; 10 g glucose per liter of distilled water⁴ pH 7.5 and incubated at 30°C for 24 h in an incubator shaker (150 rpm). Four ml of seed culture ($OD_{600} \approx 12$) was added to production media containing 26.0 g peptone; 9.0 g yeast extract; 5.0 g arabinose; 3.0 g sodium chloride; 6.6 g NH_4HPO_4 ; 7.0 g beef extract²³, N-methylacetamide (70 mM as non substrate inducer at 0 h) per liter of distilled water at pH 9.0. These flasks were incubated at 30°C for 24 h at 150 rpm in an incubator shaker.

Acyltransferase assay

The acyltransferase activity was determined spectrophotometrically by modified method described by Brammar and Clarke⁸. The reaction mixture (2 ml) contained (if not otherwise mentioned) K-phosphate buffer (0.2 M, pH 7.5), 100 mM of acetamide, 200 mM of hydroxylamine-HCl (freshly neutralized with 10.0 N NaOH) and resting cells. This reaction mixture was incubated at 60°C for 5 min in a water bath shaker and the reaction was stopped by the addition of 4 ml of

FeCl₃ reagent [6.0 % (w/v) in 2.0 % HCl (v/v)]. A control was also prepared by omitting the resting cell suspensions, which was added after the reaction was stopped to consider the auto conversion of acetamide into racemic mixture of hydroxamic acid. The reaction mixture was centrifuged at 10,000 g for 5 min, discarded the pellet and clear supernatant was collected for estimation of hydroxamic acid. The absorbance was read at 500 nm. One unit (U) of acyltransferase activity was defined as that amount of enzyme which catalyzed the release of one micromole of acetohydroxamic acid (AHA) per min under assay conditions.

Optimization of culture conditions for acyltransferase production

Preliminary experiment one variable at a time (OVAT) was designed for maximum production of acyltransferase activity of amidase by optimizing culture conditions. A number of variables such as media (M1-M13), various organic and inorganic nitrogen sources (3.0 w/v), pH (5-11), temperature (25-60°C), inducer (70 mM different types of amides and nitriles), were taken into consideration for their effect on the production of acyltransferase activity.

Optimization of acyltransferase production using statistical designs of RSM Plackett-Berman Design

Plackett-Burman design (Design Expert software 9.0.3) was used for screening medium components with respect to their main effects. The following range of media components were taken, acetamide (1-5 %), glucose (0.1-0.4 %), yeast extract (0.5-3 %), sodium chloride (0.1-0.6 %), pH (5-9), temperature (20-40°C), inoculum (2-6 %) and inducer (50-90 mM) for acyltransferase production from *Rhodococcus pyridinivorans*. Experiments were performed with various combination of high and low level of variables and analyzed for their effect on acyltransferase production. Pareto chart was constructed to find out the positive factors.

Central composite Design

Central composite Design (CCD) experiments were designed to work out optimum values of pH,

temperature, inoculum and inducer which showed positive effect on acyltransferase production. Second-order polynomial coefficient were also calculated and analyzed by using Design Expert software 9.0.3.

Acyltransferase production and growths profile of *Rhodococcus pyridinivorans*

The growth curve and acyltransferase activity profile of *Rhodococcus pyridinivorans* was studied in 50 ml production medium supplemented with 70 mM acetonitrile (as it emerged as the best inducer).

Two ml of samples were withdrawn after an interval of 3 h up to 72 h. The acyltransferase activity was assayed and cell mass (mg dcw ml⁻¹) was determined turbidometrically (OD₆₀₀).

Hyper induction of acyltransferase in *Rhodococcus pyridinivorans* by application of inducer in different feedings

Once the time of incubation for maximum enzyme production was achieved, a study was carried out to find if the application of inducer in different feedings could enhance the enzyme activity production of enzyme. In order to investigate the effect of step feeding of inducer seven different combinations were designed.

Comparison between optimized and un-optimized conditions

Once the culture conditions for maximum acyltransferase production were optimized, a comparative analysis was made between the un-optimized i.e. initial culture conditions and the optimized conditions to evaluate the outcome of this study.

Results

Screening of media

Rhodococcus pyridinivorans was grown in different media and acyltransferase activity was maximum (146.23 ± 2.303 U mg⁻¹ dcw) in M13 medium with growth production of 16.61 ± 0.140 mg dcw ml⁻¹. *Rhodococcus pyridinivorans* exhibited good growth production in all the media except M10, but production of acyltransferase activity was zero in M1, M5, M9, and M10 media (Table 1).

Table1. Media optimization for production of acyltransferase from *Rhodococcus pyridinivorans*

| Medium No. | Enzyme activity (U mg ⁻¹ dew) | Growth (mg dew ml ⁻¹) | References |
|------------|---|--------------------------------------|---------------------------------------|
| M1 | 0.00 ± 0.00 | 11.82 ± 0.150 | GY Medium ¹⁶ |
| M2 | 115.02 ± 2.380 | 13.24 ± 0.200 | Modified Nutrient Broth ³¹ |
| M3 | 133.14 ± 3.140 | 17.18 ± 0.384 | Modified Nutrient Broth ²⁶ |
| M4 | 99.80 ± 4.230 | 11.55 ± 0.408 | Modified Nutrient Broth ⁷ |
| M5 | 0.00 ± 0.00 | 8.17 ± 0.210 | Nutrient Broth |
| M6 | 96.20 ± 1.034 | 15.79 ± 0.112 | Enriched Nutrient Broth ⁴ |
| M7 | 98.34 ± 3.202 | 12.88 ± 0.532 | MY Medium ³⁷ |
| M8* | 124.98 ± 1.125 | 13.52 ± 0.280 | Control Medium ²³ |
| M9 | 0.00 ± 0.000 | 7.73 ± 0.438 | Modified MY Medium ¹⁶ |
| M10 | 0.00 ± 0.000 | 2.53 ± 0.390 | Modified MY Medium ¹⁶ |
| M11 | 20.50 ± 1.20 | 8.17 ± 0.180 | APY Mineral Salt Medium ⁵ |
| M12 | 108.80 ± 1.115 | 12.67 ± 0.385 | Modified Nutrient Broth ³¹ |
| M13 | 146.23 ± 2.303 | 16.61 ± 0.140 | Modified Nutrient Broth ²⁶ |

*Control

Effect of nitrogen sources on acyltransferase production

Rhodococcus pyridinivorans was grown in medium containing different organic and inorganic nitrogen sources (3.0 % w/v) viz. peptone, tryptone, casein enzyme, soyatone, casein acid acetamide, gelatin, urea, NH₄NO₃, (NH₄)₂SO₄, Ca(NO₃)₂, (NH₄)₂HPO₄. A control was also set without addition of nitrogen source. In presence of acetamide, *Rhodococcus pyridinivorans* showed significant increase in acyltransferase activity (409.97 ± 6.00 Umg⁻¹dew) while good cell growth (26.46 ± 0.33 mg dcw ml⁻¹) was observed in control (without addition of nitrogen source) but with very less enzyme activity (Fig. 1). However, peptone, casein enzyme, soyatone also showed good growth and production but acetamide was proved an important factor in terms of acyltransferase activity by *Rhodococcus pyridinivorans* and was selected for further study.

Effect of production pH

The pH of the culture medium M13 was varied from 5.0-10.0. The effect of pH of medium on growth and acyltransferase activity by *Rhodococcus pyridinivorans* is shown in Fig. 2. The growth of *Rhodococcus pyridinivorans* vary

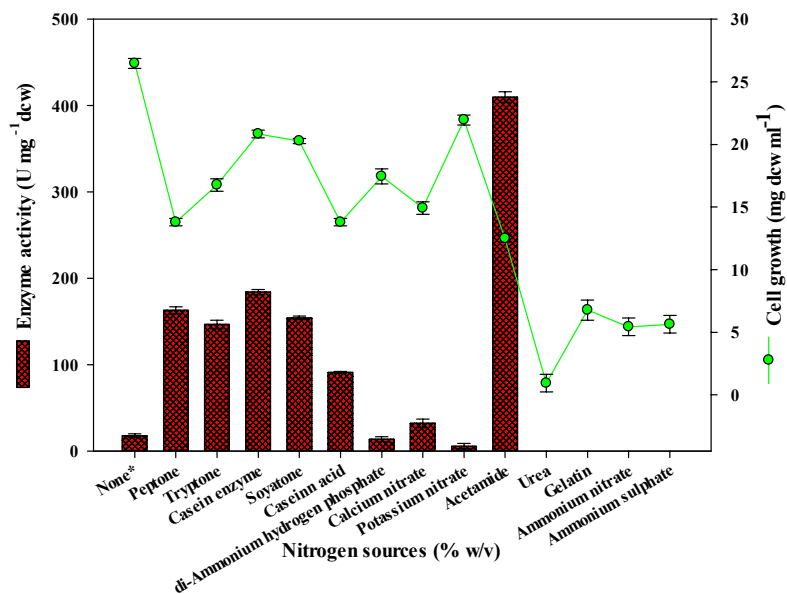
with the change in pH range of 5.0-11.0. High pH (10.5-11.0) of medium had negative effect on growth and production of acyltransferase activity of the organism. At pH 7.5 and 8.0 the growth of *Rhodococcus pyridinivorans* was quite comparable but produced higher acyltransferase activity (432.35 ± 3.23 Umg⁻¹ dcw) at pH 8.0.

Effect of production temperature

Rhodococcus pyridinivorans was grown at different temperature ranging from 25-60°C (Fig. 3) and highest acyltransferase activity was recorded at 30°C (435.93 ± 3.10 Umg⁻¹dew) with better growth (12.56 ± 0.300 mg dcw ml⁻¹). As the organism does not grow beyond 40°C thus, acyltransferase activity declined due to denaturation of required biological components. This organism was thus mesophilic in its requirements of temperature for the growth as well as for production of acyltransferase activity.

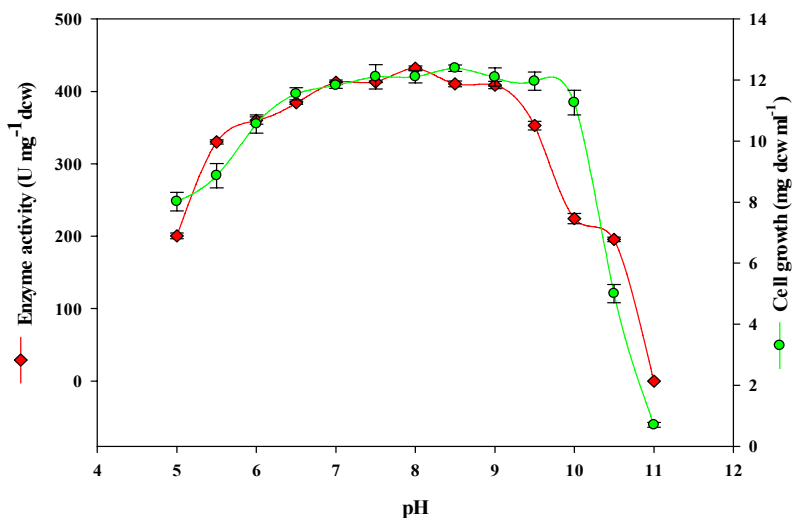
Effect of different types of inducers

The induction of acyltransferase activity of *Rhodococcus pyridinivorans* was done by adding different nitriles and amides (70 mM) in the culture medium. The acyl transfer activity was induced in presence of the nitriles as well as



*Control

Fig. 1. Effect of nitrogen sources on production of acyltransferase from *Rhodococcus pyridinivorans*



*Control

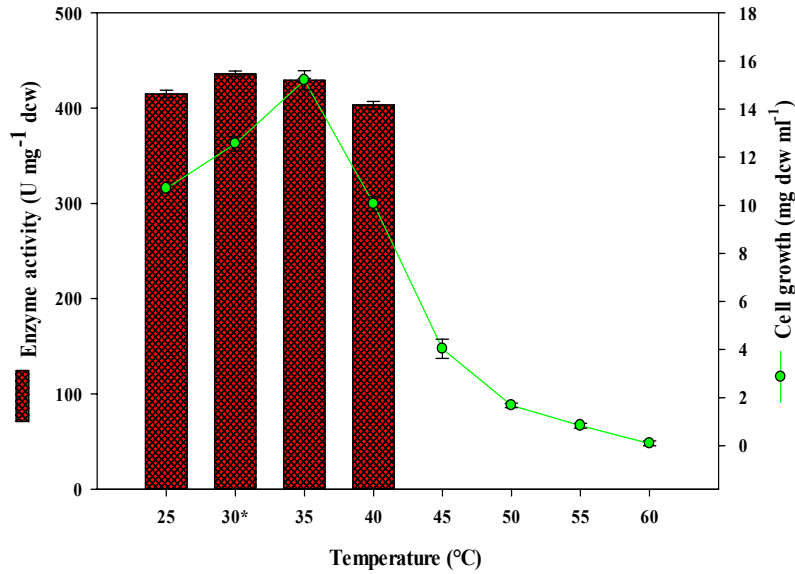
Fig. 2. Effect of medium pH on production of acyltransferase from *Rhodococcus pyridinivorans*

amides with enhancement of growth too and acetonitrile emerged to be very effective inducer. An acyltransferase activity of 448.44 ± 5.69 U mg⁻¹ dcw was observed when acetonitrile was used as inducer while maximum growth production (17.32 ± 0.10 mg dcw ml⁻¹) was observed with acetamide (Fig. 4).

Screening of important factors using Plackett-Burman design

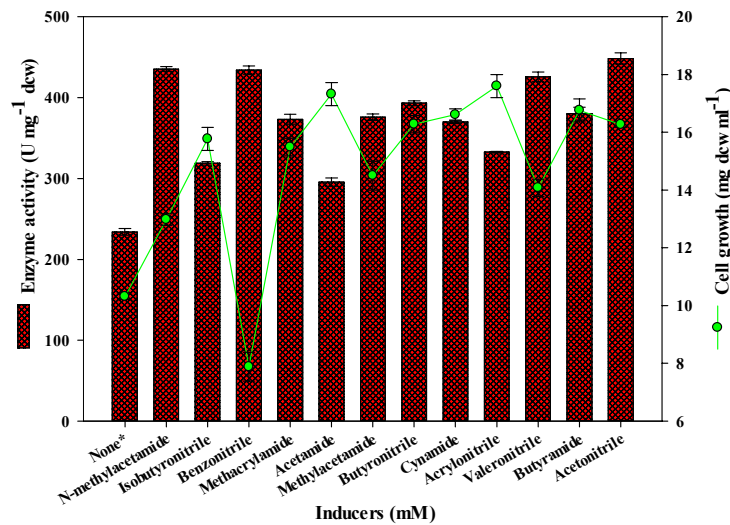
The effects of eight independent variables (ac-

etamide, glucose, yeast extract, sodium chloride, pH, temperature, inoculum and inducer) were observed on production of acyltransferase and study was carried in 12 runs using Plackett-Burman design. Fig. 5 showed that out of eight selected independent variable four variable i.e. pH, temperature, inoculum size and inducer have positive effect on enzyme production. ANOVA analysis of Plackett-Burman results was performed and a first-order polynomial equation was derived to explain the effect of various variables on



*Control

Fig. 3. Effect of temperature on production of acyltransferase from *Rhodococcus pyridinivorans*



*Control

Fig. 4. Effect of different types of inducers on production of acyltransferase from *Rhodococcus pyridinivorans*

acyltransferase production.

$$R1 = +206.22 * A - 95.17 * C + 7.24 * E + 42.33 * F + 18.46 * G + 51.30 * H + 88.18 * J + 13.35 * K + 33.96 * L$$

This equation showed the magnitude of effect of different independent variables. R1 is the activity (U mg⁻¹ dcw) and A, C, E, F, G, and H are the coded value of different variable, i.e. A-acetamide, C-yeast extract, E-pH, F-temperature, G-inoculum size and H-inducer. Effective levels of various components (pH, temperature, inoculums and inducer) were further investigated designing central composite design.

Central composite design (CCD)

Central composite design was used for the determination of optimum level and combined effect of four variables having positive effect (pH, temperature, inducer and inoculum size). The results of CCD were fitted into second order polynomial equation for the prediction of response on the bases of coded value. For response surface designs, the perturbation plot [Fig. 6(A)] shows how the response changes as each factor moves from the chosen reference point, with all other factors held constant at the reference value. De-

Pareto chart

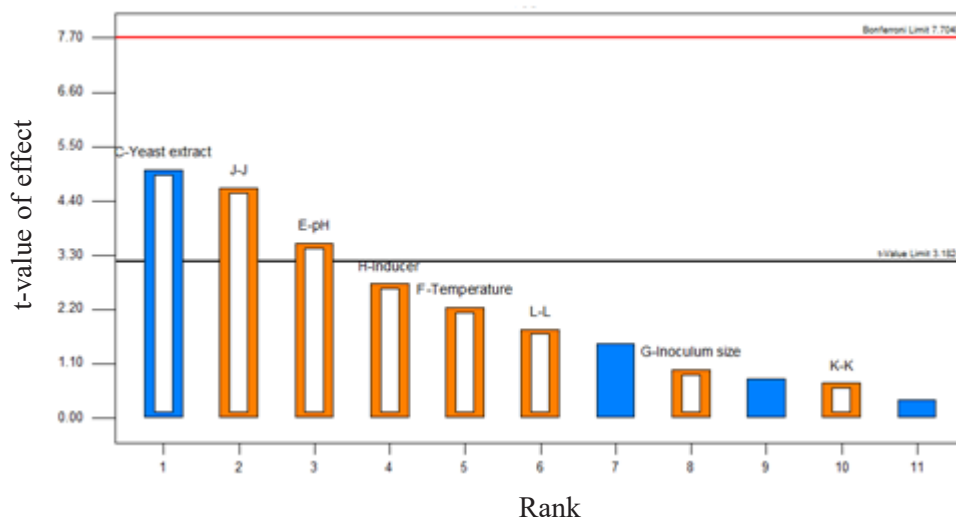
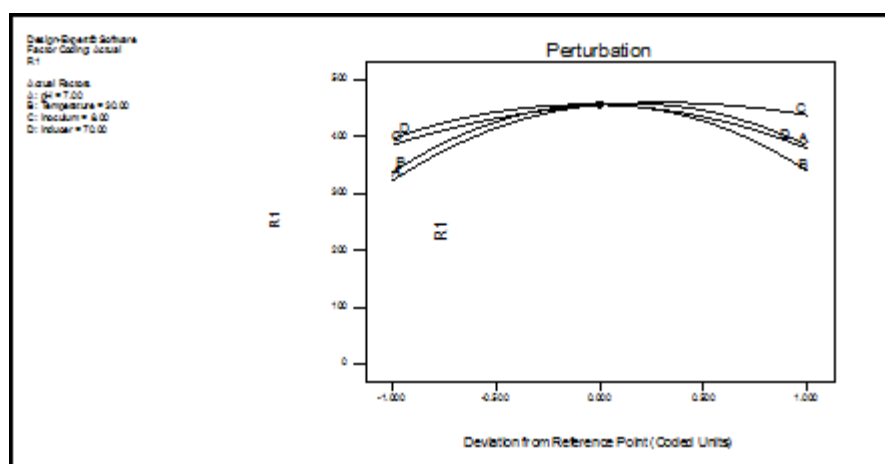
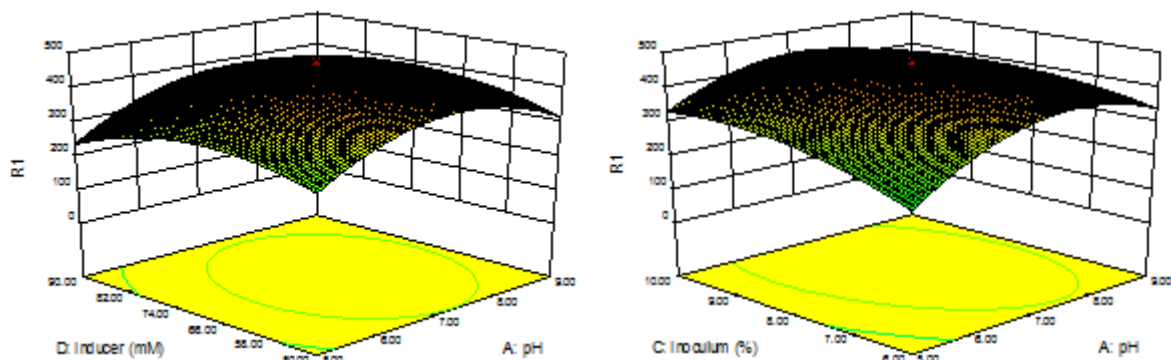


Fig. 5. Pareto chart showing the positive and negative effects of selected variables



(A)



(a)

(b)

Fig. 6 (i). Central composite design: [A] Perturbation plot of the four positive variable on response i.e. pH, temperature, inoculum size, inducer.[B] (a) inducer and pH (b) inoculum size and pH

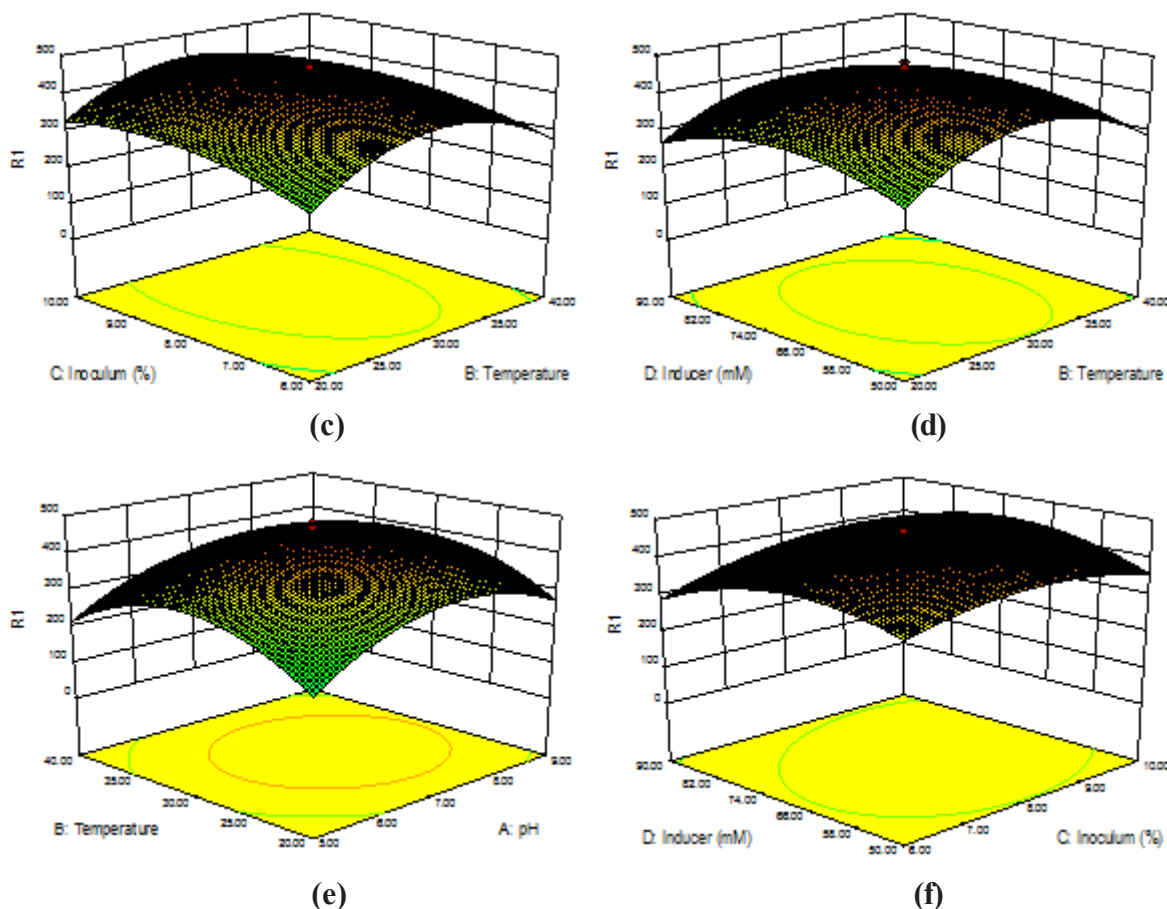


Fig. 6 (ii). Central composite design: (c) inoculum and temperature (d) inducer and temperature (e) temperature and pH (f) inducer and inoculum size on response (enzyme activity)

sign-Expert sets the reference point default at the middle of the design space (the coded zero level of each factor).

Based on the results of Plackett–Burman design, the effect of variable pH, temperature, inoculums and inducer on the production of acyltransferase were examined at four levels.

The following regression equation was derived after the analysis of variance.

$$R1 = +464.71 + 30.61 * A + 0.94 * B + 26.00 * C - 8.87 * D - 7.52 * AB - 26.30 * AC + 9.18 * AD - 8.09 * BC - 7.95 * BD + 20.92 * CD - 102.39 * A^2 - 117.37 * B^2 - 44.50 * C^2 - 68.26 * D^2$$

Where R1 is the response value (acyltransferase production) and A, B, C and D is the coded value of pH, temperature, inducer and inoculum size respectively.

Analysis of the variance of the model

Adequacy of the model was checked using analy-

sis of variance for Response Surface Quadratic model and the result was presented in Table 2. The Model F-value of 88.25 implies the model is significant. There is only a 0.01 % chance that an F-value this large could occur due to noise. “Values of “Prob > F < 0.0001 which is less than 0.0500 indicate that model terms are significant. In this case A, C, AC, CD, A², B, C, D² are significant model terms. The “Lack of Fit F-value” of 1.18 implies that the lack of fit is not significant relative to pure error; hence, non-significant lack of fit is good because we want the model to fit.

The value of determination coefficient R² of 0.9880 for response R1, implies that 98.8% of data variability can be explained by model which is closer to 1 denotes stronger correlation between the observed and predicted responses. The Predicted R-Squared of 0.9463 is in reasonable agreement with the Adjusted R-Squared of 0.9768 i.e. the difference is less than 0.2. Adeq Precision

Table 2. Analysis of the variance (ANOVA) of the model

| Analysis of variance table [Partial sum of squares - Type III] | | | | | | |
|--|----------------|----|-------------|---------|------------------|-----------------|
| Source | Sum of Squares | Df | Mean Square | F Value | p-value Prob > F | |
| Model | 6.98E+05 | 11 | 49855.78 | 88.25 | < 0.0001 | significant |
| A-pH | 22482.43 | 1 | 22482.43 | 39.8 | < 0.0001 | |
| B-Temperature | 21.36 | 1 | 21.36 | 0.038 | 0.8484 | |
| C-Inoculum | 16224 | 1 | 16224 | 28.72 | < 0.0001 | |
| D-Inducer | 1890.02 | 1 | 1890.02 | 3.35 | 0.0873 | |
| AB | 905.71 | 1 | 905.71 | 1.6 | 0.2248 | |
| AC | 11070.2 | 1 | 11070.2 | 19.6 | 0.0005 | |
| AD | 1349.46 | 1 | 1349.46 | 2.39 | 0.143 | |
| BC | 1048.46 | 1 | 1048.46 | 1.86 | 0.1932 | |
| BD | 1012.51 | 1 | 1012.51 | 1.79 | 0.2006 | |
| A ² | 2.88E+05 | 1 | 2.88E+05 | 509.04 | < 0.0001 | |
| B ² | 3.78E+05 | 1 | 3.78E+05 | 668.9 | < 0.0001 | |
| C ² | 54320.01 | 1 | 54320.01 | 96.15 | < 0.0001 | |
| D ² | 1.28E+05 | 1 | 1.28E+05 | 226.2 | < 0.0001 | |
| Residual | 8473.85 | 15 | 564.92 | | | |
| Lack of Fit | 5958.86 | 10 | 595.89 | 1.18 | 0.4516 | not significant |
| Pure Error | 2514.98 | 5 | 503 | | | |
| Cor Total | 7.07E+05 | 29 | | | | |

measures the signal to noise ratio. A ratio greater than 4 is desirable; our ratio of 28.048 indicates an adequate signal. The low CV (12.52) denotes the experiment performed was reliable. The p values denotes the significance of coefficient and also important in understanding the mutual interactions between the variables. Three-dimensional plots were generated for regression analysis of CCD design of pair-wise combination of three factors for acyltransferase production. The effects of the independent variables and combined effects of each independent variable upon the response variable were described by plotting 3d graphs [Fig. (i, ii) 6(B) a.b.c.d.e.f].

Validation of model

The maximum experimental response for acyltransferase production was 480.86 ± 1.230 U mg⁻¹ dcw while the predicted value 474.71 U mg⁻¹ dcw indicating close agreement between them (Fig. 7). Perturbation plot (Fig. 6 (i) (A)) showed the optimum value for variables pH 7.0, temperature 30°C, inoculums 8%, inducer 70 mM per 1000

ml. The plot indicates that pH was the most influential component while others showed least influence on acyltransferase activity. The model was validated by repeating the experiment under optimized conditions, which resulted into 474.66 ± 2.01 U mg⁻¹ dcw (predicted response 464.71 U mg⁻¹ dcw, Fig. 7) activity and proved the validity of the model. RSM was able to enhance the acyltransferase activity of *Rhodococcus pyridinivorans* from 448.44 ± 7.00 U mg⁻¹ dcw to 474.8 ± 2.01 U mg⁻¹ dcw. Thus 1.06 fold increase in acyltransferase activity was observed.

Acyltransferase production and growth profile of *Rhodococcus pyridinivorans*

Rhodococcus pyridinivorans was grown in medium being optimized and activity was assayed after every 3 hours followed up to 72 hours. The best growth (24.92 ± 2.00 mg dcw ml⁻¹) recorded at 30 hours while maximum enzyme activity (499.03 ± 4.00 U mg⁻¹ dcw) was recorded after 18 hours of incubation at 30°C (Fig. 8), whereas pH of the culture increased with the growth of

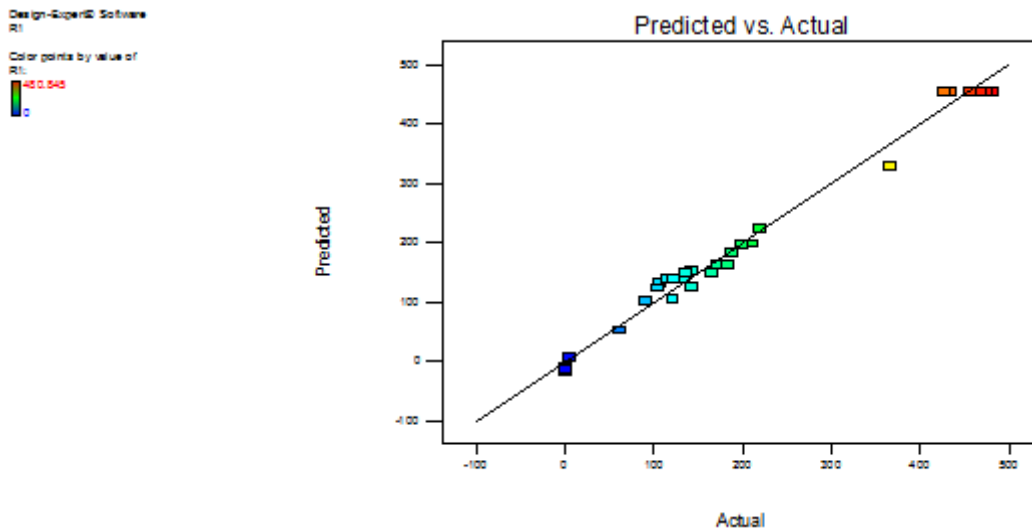
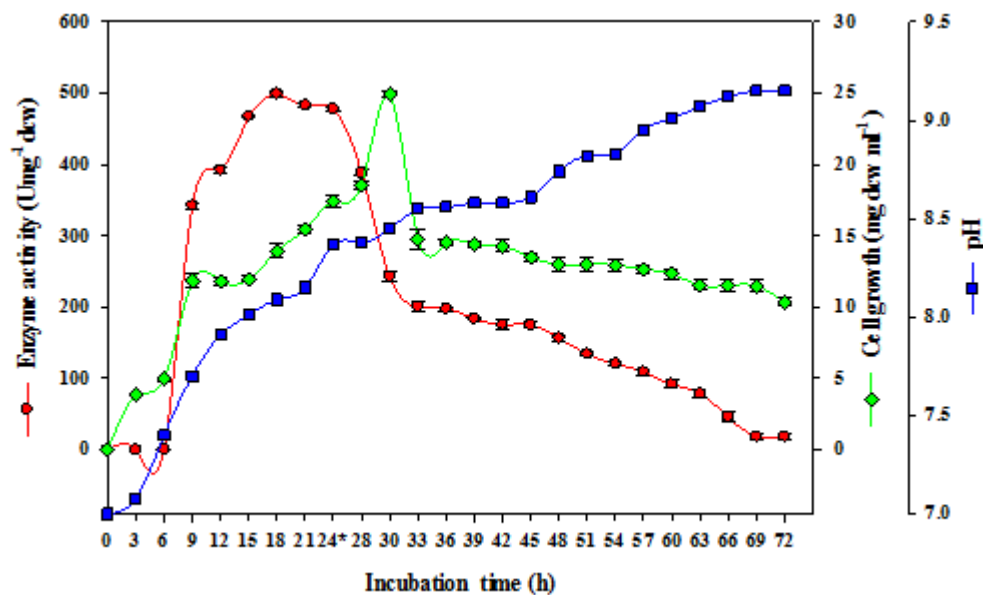


Fig. 7. Graph showing experimental validation of model with actual and predicted level of acyltransferase production from *Rhodococcus pyridinivorans*



*Control

Fig. 8. Acyltransferase production and growth profile of *Rhodococcus pyridinivorans*

the bacterium in the presence of inducer which means that ammonia was being produced as the organism utilized the organic and inorganic nitrogen sources.

Hyper induction of acyltransferase in *Rhodococcus pyridinivorans* by multiple feeding of inducer

Out of the seven different combinations designed (Table 3) for the application of inducer, the step-

feeding of inducer at 0, 6 and 12 h of incubation showed maximum enzyme activity (519.03 ± 1.093 U mg⁻¹ dcw) in combination number 5. Multiple feedings of inducer resulted in enhanced induction of enzyme but there was not much variation observed in growth of the organism with a single feed.

Comparative analysis of initial and optimized culture conditions

A comparison between the un-optimized i.e. ini-

Table 3. Hyper induction of acyltransferase in *Rhodococcus pyridinivorans* by application of inducer in different feedings

| Combination No. | Inducer (acetonitrile, 70 mM) | Acyltransferase activity (U mg ⁻¹ dcw) | Growth mg dcw ⁻¹ ml |
|-----------------|-------------------------------|---|--------------------------------|
| 1 | *Only once at 0h for 18hrs | 498.59 ± 3.34 | 17.01 ± 0.134 |
| 2 | 0h+6h | 516.03 ± 2.65 | 17.05 ± 0.095 |
| 3 | 0h+12h | 510.46 ± 2.58 | 17.40 ± 0.023 |
| 4 | 0h+18h | 499.32 ± 4.02 | 17.02 ± 0.350 |
| 5 | 0h+6h+12h | 519.03 ± 1.03 | 17.89 ± 0.045 |
| 6 | 0h+6h+12h+18h | 513.49 ± 2.42 | 17.73 ± 0.256 |
| 7 | 0h+12h+18h | 508.24 ± 2.56 | 17.32 ± 0.135 |

*Control

tial culture conditions and the optimized conditions showed that the study was quite successful and almost 4.02 fold increase in acyltransferase production was observed when *Rhodococcus pyridinivorans* was cultivated in optimized conditions i.e. acetamide 3.0 %, yeast extract 1.5 %, NaCl 0.5 %, glucose 0.2 %, pH 7.0, temperature 30°C, 8 % inoculum size and acetonitrile as non substrate inducer (70 mM) for 18 h (519.03 ± 4.00 U mg⁻¹ dcw) as compared to un-optimized conditions (124.31 ± 1.125 U mg⁻¹ dcw).

Discussion

Due to the rising interest in sustainable development and operation under milder conditions, biotransformation processes are gaining importance in the synthesis of various chemicals and drug intermediates. The present study was focused on optimization of culture parameters to get maximum production of acyltransferase from *Rhodococcus pyridinivorans* using statistical experimental design. Among the different media used, Medium No. 13 containing tryptone 3.0 %, NaCl 0.5 %, glucose 0.2 %, yeast extract 1.5 %²⁶, proved to be the best medium for the production of acyltransferase. In earlier investigations^{9, 14, 21} modified mineral salt medium have been used for the production of nitrile hydrolyzing enzymes whereas Krieg *et al.*¹⁸ used vitamin solution (2.5 ml/l) and trace element solution (0.8 ml/l) along with KH₂PO₄ (0.33 %), K₂HPO₄ (0.08 %), NaCl (0.1 %), CaCl₂.2H₂O (0.005 %) and MgSO₄.7H₂O (0.03 %) for the production of amidase from

Variovorax paradoxus showed significant increase in acyltransferase activity when medium was supplemented with acetamide as source of nitrogen. Organic nitrogen sources were found to be more favorable in terms of acyltransferase activity and biomass production of *Rhodococcus pyridinivorans*. This might be due to existence of ammonium form (NH₄⁺) under acidic conditions which may leads to inhibition of growth of culture and synthesis of acyltransferase. Black *et al.*⁷ used 12.5 g/l peptone for the production of amidase whereas some workers^{4, 17, 37} have used 5.0 g/l peptone for amidase production. Pandey *et al.*²³ used 0.66 % (w/v) (NH₄)₂HPO₄ as nitrogen source for amidase production from *Rhodococcus pyridinivorans*. Similarly, Bhalla *et al.*⁵ also used 0.66 % (w/v) (NH₄)₂HPO₄ in medium for amidase production from *Rhodococcus rhodochrous* PA-34. The variation in pH alters acid-base equilibrium, which greatly affects the uptake of nutrients in the medium. Drastic variation in pH may harm the microbial cells by disrupting plasma membrane thus inhibit the activity of enzyme. However, this organism efficiently produces growth and acyl transfer activity around neutral pH (8.0) and afterwards decrease in pH was observed. Majority of nitrile hydrolyzing organisms reported so far exhibited growth and enzyme production around neutral pH. Kelly and Clarke¹⁵ observed maximum growth of *Pseudomonas aeruginosa* and amidase production at pH 7.2 where as it was 7.0 in case of *Brevibacterium* sp. R312³⁴. Bhalla *et al.*⁴ have

reported maximum amidase production when *Rhodococcus* sp. NHB-2 was cultivated at pH 5.5 and 8.0 and suggested that there might be more than one type of amidase in this organism. Black *et al.*⁷ have reported an optimum pH of 8.0 for the growth and amidase production in case of *Xanthobacter agilis*. Prasad *et al.*²⁸ have reported that optimum pH for amidase production from *Kluyveromyces thermotolerans* MGBY 37 was 6.2. Increase in acyltransferase activity and cell growth was observed with increase in temperature up to 30°C and 35°C, respectively and thereafter it declines. Beyond 40°C protein denaturation occurred causing the slow metabolism of cells, which ultimately affects the cell growth and productivity and showing its mesophilic nature. Similarly, *Alcaligenes* sp. MTCC 10674 produced maximum acyl transfer activity at 30°C⁶ as reported for most of other amidase producing mesophilic organisms⁴.

Generally, acyltransferase activity of amidase is produced constitutively by many microbes^{2,18,31} whereas in some microorganisms it is induced by amides and nitriles^{2,10}. The acyltransferase activity of *Rhodococcus pyridinivorans* is inducible as well as constitutive and acetonitrile (70mM) proved to be the best inducer. A comparable activity was also observed with N-methylacetamide, valeronitrile and benzonitrile. Bhatia *et al.*⁶ and Sharma *et al.*³² respectively used isobutyronitrile (0.4% v/v) and formamide (0.3%) for acyltransferase production from *Alcaligenes* sp. MTCC 10674 and *Geopallidus pallidus* BTP-5x MTCC 9225.

Based on results of one factor at time, the optimized values was taken into consideration for further production of acyltransferase by adopting statistical design approach using response surface methodology. Experiments were performed at various combinations of high and low values of process variables and analyzed for their effects on the response of process. For improving the production of acyltransferase from *Rhodococcus pyridinivorans* eight independent variables were screened by using Plackett-Burman design in which four positive variables (pH, temperature, inducer and inoculum) were found to be most influential media components. After this statistical

optimization acyltransferase production was found to increase according to prediction value generated by model. Acyltransferase production following OVAT was about 448.44 ± 7.00 U mg⁻¹ but following to prediction of CCD it was about 474.8 ± 2.01 U mg⁻¹ dcw. According to Vaidya *et al.*³⁵ sorbitol, yeast extract, meat peptone and acetamide the most effective factors for production of amidase (acyltransferase) from *Rhodococcus erythropolis* MTCC 1526. The model F-value of 11.04 indicating the model was significant. R² value of 0.912 indicating that 91.2% of variability in the response could be explained by the model³⁵.

The induction of amidase for acyl transferase activity in *Rhodococcus pyridinivorans* was also observed in the log phase of growth which starts after 6 h of incubation and maximum acyl transferase activity was achieved after 18 h with maximum growth at 30 h. A decrease in activity and cell growth after optimal value could be either due to decrease in nutrient availability in medium or catabolite repression of enzyme. Final pH of the culture increased with the growth of the bacterium which means that ammonia was being produced as the organism utilizing the nitrogen sources. However, *R. rhodochrous* J1¹⁷ and *K. pneumoniae*²¹ have been reported to produce maximum amidase after 48 h of incubation while amidase of *Geobacillus pallidus* BTP-5x b MTCC 9225 was 20 h of incubation³².

Comparable activity was also observed in combinations number 2 (516.03 ± 2.65) and 6 (513.49 ± 3.42) when multistep feeding of inducer was given to culture medium, although the activity is highest in combination number 5 (519.03 ± 1.093 U mg⁻¹ dcw), hence considered optimum for further studies. Pandey *et al.*²³ have design 16 different combinations for the application of inducer in multiple feedings, out of sixteenth combinations the culture medium in which inducer (N-methylacetamide) was added to 0, 24 and 30 h of incubation showed maximum acyltransferase activity of *Bacillus* sp. APB-6.

Conclusion

To sum up, the response surface model has been proved to be a valuable tool for predicting and

optimizing the cultivation conditions to maximize the acyltransferase production. Response surfaces curves were very helpful in visualizing the main effects and interaction of factors. The validity of the model was confirmed by close agreement between experimental and predicted value. The optimum culture medium obtained in this experiment has given a base for further study. Medium optimization both by conventional method and statistical method effectively enhanced acyltransferase production upto 4.2 fold.

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Conflict of interest

Authors declare that there is no conflict of interest.

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