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Optimization of Media Components for Production of α-L-rhamnosidase from *Clavispora lusitaniae* KF633446

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A B S T R A C T

Rhamosidase producing yeast strain 84 was isolated from whey beverage and identified as *Clavispara lusitaniae* KF633446. The effect of different carbon sources (rhamnose, glycerol, lactose, fructose, glucose and sucrose), nitrogen sources (yeast extract, peptone, ammonium chloride, ammonium sulphate, urea and casein), temperature (10-60°C) and pH (3-8) were studied to optimize the production of rhamnosidase enzyme from *Clavispara lusitaniae* 84. Further, a multivariate response surface methodology evaluated the effects of different factors on enzyme activity and optimized enzyme production. The fit of the model (R^2= 0.409479) was found to be significant. Results indicated that yeast showing maximum rhamnosidase activity (0.106 IU mL^{-1}) in presence of rhamnose (0.6% w/v), yeast extract (0.4% w/v), temperature (35±5 °C) and pH (4) in the minimal medium supplemented with naringin (0.2% w/v).

Introduction

Many citrus juice processing has commercial restrictions due to bitter taste by chemical naringin. Many techniques are used to reduce naringin such as adsorptive debittering (Fayoux *et al.,* 2007), enzymatic hydrolysis (Puri and Kalra, 2005), poly-styrene divinyl benzene styrene resin treatment and β-cyclodextrin treatment (Mongkolkul *et al.,* 2006). These techniques have limitations in altering nutrient composition by chemical reactions or removal of nutrients, flavor and color etc. In comparison, the enzymatic debittering technology is regarded as the most promising method with the advantages of high specificity and efficiency and a convenient operation for removing the bitterness in large-scale commercial production (Yadav *et al.,* 2010).

α-L-Rhamnosidase is used for debittering the citrus juice by hydrolyzing bitter naringin to nonbitter prunin and rhamnose, resulting in a taste improvement of citrus juice and derived beverages. α-L-Rhamnosidase is produced by many microorganisms mainly filamentous fungi (*Aspergillus, Circinella, Eurotium, Fusarium, Penicillium, Rhizopus* and *Trichoderma*) (Scaroni *et al.,* 2002). In case of yeast strains, low levels of rhamnosidase activity have been reported (Rodriguez *et al.,*

2004). Some yeast like *Sacchromyces cerevisiae*, *Hanshula anomala*, *Debaryomyces polymorphus* and *Pichia angusta* X 349 (Yanai and Sato, 2000) produce low level of α-L-hamnosidase activity (McMahon *et al.,* 1999). Using rhamnosidase producing micro-organism, the process of debittering is economically viable and more cost effective than other processes.

Media components play an important role in enhancing the enzyme production. Rhamnosidase production mainly depends on the inducer, carbon and nitrogen source given to the microorganism. Reported inducers for naringinase production are rhamnose (Thammawat *et al.,* 2008), hesperidin (Fukumoto and Okada, 1973), naringin (Bram and Solomons, 1965; Puri *et al.,* 2008) and citrus peel powder (Puri *et al.,* 2011). Temperature is one of the most important variable affecting enzyme deactivation by weakening non-covalent interactions that stabilize the protein structure and leading to unfolding and subsequent changes that reduce the catalytic activity (Klibanov, 1983), change in the pH value can also irreversibly change the protein structure by alteration of the charge of the amino acid responsible for maintenance of the secondary and tertiary structure (Bisswanger, 1999). So, the optimization of physical and nutritional conditions is very essential.

Optimizing the affecting parameters by statistical experimental designs can eliminate the limitations of a single factor optimization process collectively (Montogomery, 2000). Response surface methodology (RSM) is a useful statistical technique for the investigation and optimization of complex processes. It uses quantitative data from an appropriate experimental design to determine and simultaneously solve a multivariate equation (Rastogi *et al.,* 2010). Central composite design (CCD) is a widely used response surface design when the experimental region is defined by the upper and lower limits of each factor and not extended beyond them (Neter *et al.,* 1996). A combination of factors generating a certain optimal response can be identified. Also, significant interactions between variables can be identified and quantified by this approach (Vishwanatha *et al.,* 2010).

Therefore, the paper aimed to optimize the media composition to increase rhamnosidase production by *Clavispora lusitaniae* KF633446.

Materials and Methods

Microorganism and Growth Conditions

Yeast strain (84) producing rhamnosidase enzyme was isolated from whey beverage and identified as *Clavispora lusitaniae* (accession number KF633446) on the basis of morphological, biochemical and 18S rDNA sequence analysis. The minimal medium (g/l: glucose 5.0, Na_2HPO_4 6.0, KH_2PO_4 3.0 g L^{-1}, NH_4Cl 1.0, NaCl 0.5, $MgSO_4$ 0.12, $CaCl_2$ 0.1, naringin 2 and pH 6) was used for growth and enzyme production. 50 mL of the resultant medium in Erlenmeyer flask (100 ml) was aerobically cultured at 28±2 °C for 1-4 d on a rotary shaker at 150 rpm. After centrifugation (12,000 × *g*, 4 °C, for 15 min), the supernatant was collected to measure rhamnosidase activity.

α-L- Rhamnosidase enzyme assay

The α-L-rhamnosidase activity (RA) was determined using p-nitrophenyl-α-L-rhamnoside (p-NPR, Sigma) as the substrate (Romero *et al.,* 1985). The reaction mixture consisted of 0.1 mL of 4.8 mM p-NPR solution, plus 0.19 mL of 50 mM sodium acetate/ acetic acid buffer, pH 5.0 and 10 μL of enzyme or buffer (for the blank) and was

incubated at 50 °C. Aliquots of 50 µL from the reaction mixture were removed every 2 min and placed into 1.5 mL of 0.5 M NaOH. These aliquots were kept in an ice bath until the absorbance was measured at 400 nm (Rajal *et al.,* 2009). One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 µmol of p-nitrophenol per minute.

Screening of media components for optimization α-L- rhamnosidase production

The media composition was optimized following 'one-at-a-time' approach to increase α-L-rhamnosidase production. Six different carbon sources (glucose, lactose, sucrose, glycerol, fructose and rhamnose) were added individually at 5 gL^{-1} in the minimal medium containing 0.2% naringin. Four organic nitrogen sources (1 gL^{-1} peptone, yeast extract, casein and urea) and two inorganic nitrogen sources (1 gL^{-1} ammonium chloride and ammonium sulphate) were also tested individually one by one keeping another factor constant. The effect of temperature in a range between 15 to 45 °C and pH in a range of 3 to 8 on enzyme activity was examined. Further, best carbon and nitrogen supplementation were used at different concentrations from 0.1 to 1%. For each parameter optimization, three sets of independent experiments were carried out and the average value was reported (Chen *et al.,* 2010; Singh *et al.,* 2012).

Experimental design

The statistical analysis of the results was performed with the aid of "Design-Expert-9.0.3" (Stat Ease, Inc., Minneapolis, USA). A 25 factorial central composite experimental design, with four factors and five replicates at the centre point, leading to a set of 30 experiments, was used to optimize the production of rhamnosidase from yeast strain 84. All the variables were taken at a central coded value considered as zero. The minimum and maximum ranges of variables investigated and the full experimental plan with respect to their values in actual and coded form are listed in Table 1. Upon completion of the experiments, the average maximum rhamnosidase yield was taken as the dependent variable or response (Y). A second-order polynomial equation was then fitted to the data by the multiple regression procedure. This resulted in an empirical model that related the response measured to the independent variables of the experiment. For a four-factor system, the model equation is:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{12} AB + \beta_{13} AC$$

$$Y = + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD$$

$$Y = + \beta_{11} A_2 + \beta_{22} B_2 + \beta_{33} C_2 + \beta_{44} D_2$$

Where: A= rhamnose, B= yeast extract, C= pH, D= incubation temperature (°C), Y= predicted response, β_0= intercept; β_1, β_2, β_3 and β_4= linear coefficients; β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34}= interaction coefficients and β_{11}, β_{22}, β_{33} and β_{44}= squared coefficients.

Analysis of variance (ANOVA) was performed. The proportion of variance explained by the polynomial models obtained was given by the multiple coefficient of determination (R_2). In order to confirm the maximum rhamnosidase production predicted by the model, three-dimensional response surface and contour presentations were plotted to find the concentration of each factor for maximum rhamnosidase production. The response surface curves were plotted for the variation in rhamnosidase yield as a function of the concentrations of one variable when all the other factors were kept at their central levels. The optimum concentration of each nutrient was identified based on the peak in the three dimensional plot (Singh *et al.,* 2012).

Statistical analysis

The data was analyzed by standard analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). Standard errors were calculated for all mean values. Differences were considered significant at the $p \leq 0.05$ level.

Results and Discussion

Screening of media components for optimization of α-L-rhamnosidase production

Effect of carbon source on α-L-rhamnosidase production

A differential response in rhamnosidase activity was obtained due to supplementation of various carbon sources. Among various carbon sources, rhamnose exhibited maximum enzyme activity i.e. 0.056 IU mL^{-1} and glucose exhibited minimum rhamnosidase activity i.e. 0.016 IU mL^{-1} after 48 h of incubation (Fig. 1A). Further, optimization of rhamnose concentration (0.1-1%-w/v), it was found that *Clavispora lusitaniae* KF633446 produced maximum enzyme (0.065 IU mL^{-1}) when grown on medium containing 0.6% rhamnose as compare to other concentrations (Fig. 1E) Yeast strains *Saccharomyces cerevisiae*, *Cryptococcus terreus*, *Pichia angusta* and *Pichia capsulate* showed low levels of α-L- rhamnosidase activity (IU mL^{-1}- 0.0137, 0.0065, 0.034 and 0.0288) in presence of rhamnose as compare to present yeast strain (Yanai and Sato, 2000).

Similar results was observed by Elinbaum *et al.,* 2002 that rhamnose could be used as an inducer in the production of *Aspergillus terreus* α-L-rhamnosidase by solid state fermentation, however they reported that naringin was a better inducer than rhamnose. Puri *et al.,* 2005 reported that naringinase activity was repressed by glucose, sucrose and lactose although these carbon sources supported excellent growth. Production of α-L-rhamnosidase by *A. kawachii* is mediated by carbon catabolite repression (Koseki *et al.,* 2008). They found that α-L-rhamnosidase production by *A. kawachii* was significantly induced in presence of 0.5% L-rhamnose, but the production was repressed in presence of 0.5% L-rhamnose supplemented with 1% glucose and enzyme was not produced when *A. kawachii* was grown on 0.5% glucose as the sole carbon source. Puri *et al.,* (2005) observed rhamnose and molasses (10 g L^{-1}) exhibited highest naringinase activity (4.6 IU mL^{-1}) in salt medium with naringenin after 8 days of fermentation (Puri *et al.,* 2005). The present study shows that yeast strain *Clavispora lusitaniae* KF633446 produces α-L-rhamnosidase in short duration fermentation (48 h) as compared to reported fungal strains. The reduction in fermentation time is important because it decreases the fermentation costs and contamination with opportunistic microorganisms in scale up process.

Effect of nitrogen source on α-L-rhamnosidase production

The effect of different nitrogen sources were tested for rhamnosidase production in minimal medium containing 0.2% naringin supplemented with 0.6% (w/v) rhamnose. Results indicated that minimal medium containing yeast extract has maximum rhamnosidase activity (0.057 IU mL^{-1}) followed by peptone (0.050 IU mL^{-1}), casein (0.047 IU mL^{-1}), urea (0.038 IU mL^{-1}), ammonium sulphate (0.035 IU mL^{-1}) and ammonium chloride (0.024 IU mL^{-1}) as a nitrogen source after 48 h of incubation (Fig. 1B). Further, among various concentration of yeast extract (0.1-1%-w/v), 0.4% (w/v) yeast extract resulted in highest rhamnosidase activity relative to other concentrations (Fig.

1F). In similar, yeast extract (Bram and Solomons, 1965) and peptone (Chen *et al.,* 2010; Puri *et al.,* 2005) were able to increased the production of naringinase enzyme. Peptone was the most effective in naringinase biosynthesis from *Aspergillus niger* (Puri *et al.,* 2005) and *Aspergillus oryzae* JMU316 (Chen *et al.,* 2010). In terms of the enzyme yield, the optimum concentration of peptone was 5 gL^{-1} and higher concentrations of peptone in the fermentation medium did not significantly increase enzyme yield (Puri *et al.,* 2005). Inorganic nitrogen sources yielded low naringinase production in shaking-flask cultures relative to organic sources (Norouzian *et al.,* 2000). Inorganic nitrogen sources could only marginally synthesize certain essential amino acids in fermentation by fungi and organic nitrogen sources were favorable for metabolite production (Hwang *et al.,* 2003; Kim *et al.,* 2003). The maximum naringinase production of *Aspergillus niger* BCC 25166 obtained by supplement of the medium with $NaNO_3$ as its nitrogen source (Thammawat *et al.,* 2008). Urea and diammonium hydrogen phosphate were inhibitory, presumably because of the release of ammonium ions (Puri *et al.,* 2005).

Effect of temperature on rhamnosidase activity

In case of temperature optimization, maximum rhamnosidase activity (0.05 IU mL^{-1}) was observed at 35±5 °C after 48 h and decreased slowly when the temperature rises (Fig. 1C). The reason for the decrease in enzyme activity above and below the 35 °C temperature may be the deactivation of enzyme by weakening of non-covalent interactions that stabilize the protein structure, leading to unfolding and subsequent changes and reduction in catalytic activity of enzyme. This suggests that the temperature for enzymatic hydrolysis of naringin and conversion of other flavonoids should be controlled at 35 °C. Optimum temperature for *Pichia angusta* (Yanai and Sato, 2000) and *Aspergillus nidulans* (Orejas *et al.,* 1999) rhamnosidases was observed at 40 °C. Yadav and Yadav (2004) found that optimum temperature of rhamnosidases from the different *Aspergillus* strains vary from 53-60 °C. The temperature optimum for naringinase activity was 50 °C for *Bacillus methylotrophicus* (Mukund *et al.,* 2014) and *Aspergillus niger* MTCC1344 (Thammawat *et al.,* 2008).

Effect of pH on rhamnosidase activity

The effect of pH on yeast rhamnosidase activity was tested in a range of 3 to 8 and best pH for rhamnosidase activity was 4 (0.05 IU mL^{-1}) then 5, 6, 7, 8 and 3 (Fig. 1D). The reason for decrease in enzyme activity above and below the pH 4 may be the change in enzymatic structure by altering charge of amino acids responsible for secondary and tertiary structure. The high response at low pH level is of great importance in fruit juice processing industry because pH of juices is often less than 5.

Additionally, low pH reduces the chances of bacterial contamination in the fruit beverages as optimum pH for the growth of most of the food borne pathogens ranges from 6.5 to 7.5.

Thus, this potential of enzyme can be utilize for the preparation of fruit beverages without preservative. In similar findings, optimum pH of rhamnosidases from *Aspergillus terreus* and *Aspergillus niger* BCC 25166 was 4 (Abbate *et al.,* 2012; Petri *et al.,* 2014; Puri and Banergee, 2000; Shamugam and Yadav, 1995). Yanai and Sato (2000) reported that enzyme purified from *Pichia angusta* showed optimum activity at pH 6 which is higher than above reported strain. Enzyme production was little affected by pH change in the range 4-6, but yields were low at pH values below 4 (Puri *et al.,* 2005).

Table.1 Variables representing medium components used in response surface methodology

Design Summary										
File Version	9.0.3.1									
Study Type	Response Surface			Runs		30				
Design Type	Central Composite			Blocks		2				
Design Model	Quadratic			Build Time (ms)		78				
Factor	**Name**	**Units**	**Type**	**Subtype**	**Minimum**	**Maximum**	**Coded**	**Values**	**Mean**	**Std. Dev.**
A	Rhamnose	G	Numeric	Continuous	-0.15	0.85	-1.000=0.1	1.000=0.6	0.35	0.227429413
B	Yeast extract	G	Numeric	Continuous	-0.15	0.85	-1.000=0.1	1.000=0.6	0.35	0.227429413
C	pH	-	Numeric	Continuous	2	6	-1.000=3	1.000=5	4	0.909717652
D	Temperature	°C	Numeric	Continuous	25	45	-1.000=30	1.000=40	35	4.548588261

Table.2 Design of RSM experiments and respective experimental and predicted α-L rhamnosidase activities

Variables under study				α-L-rhamnosidase activity (IU L^{-1})	
Rhamnose (g L^{-1})	**Yeast Extract (g L^{-1})**	**pH**	**Temperature (°C)**	**Experimental Value**	**Predicted Value**
0.1	0.1	3	30	99	96
0.6	0.1	3	30	103	102
0.1	0.6	3	30	97	109
0.6	0.6	3	30	98	93
0.1	0.1	5	30	97	101
0.6	0.1	5	30	106	104
0.1	0.6	5	30	97	99
0.6	0.6	5	30	100	106
0.1	0.1	3	40	90	92
0.6	0.1	3	40	109	91
0.1	0.6	3	40	106	98
0.6	0.6	3	40	106	102
0.1	0.1	5	40	103	101
0.6	0.1	5	40	95	103
0.1	0.6	5	40	105	95
0.6	0.6	5	40	110	99
0.35	0.35	4	35	90	109
0.35	0.35	4	35	106	103
0.35	0.35	4	35	92	92
0.35	0.35	4	35	90	95
-0.15	0.35	4	35	102	95
0.85	0.35	4	35	110	106
0.35	-0.15	4	35	110	91
0.35	0.85	4	35	96	94
0.35	0.35	2	35	109	100
0.35	0.35	6	35	96	94
0.35	0.35	4	25	103	92
0.35	0.35	4	45	109	91
0.35	0.35	4	35	110	96
0.35	0.35	4	35	91	93

Table.3 ANOVA for response surface quadratic model

Source	Sum of squares	df	Mean square	F Value	*p*-value Prob > F
Block	123.2667	1	123.266		
Model	311.45	14	22.246	0.693	0.748
A-Rhamnose	40.04167	1	40.041	1.248	0.282
B-Yeast Extract	12.04167	1	12.041	0.375	0.549
C-pH	7.041667	1	7.0416	0.219	0.646
D-Temperature	40.04167	1	40.041	1.248	0.282
AB	7.5625	1	7.562	0.235	0.634
AC	33.0625	1	33.062	1.030	0.327
AD	5.0625	1	5.062	0.157	0.697
BC	60.0625	1	60.062	1.872	0.192
BD	0.5625	1	0.562	0.017	0.896
CD	1.5625	1	1.562	0.048	0.828
A^2	46.50298	1	46.502	1.449	0.248
B^2	13.36012	1	13.360	0.416	0.529
C^2	5.002976	1	5.002	0.155	0.698
D^2	24.64583	1	24.645	0.768	0.395
Residual	449.15	14	32.082		
Lack of Fit	265.9	10	26.59	0.580	0.778
Pure Error	183.25	4	45.812		
Cor Total	883.8667	29			

AB, AC, AD, BC, BD and CD represent the interaction effect of variables A, B, C and D; A^2, B^2, C^2 and D^2 are the square effects of the variables

Table.4 Model fitting values of RSM

Model terms	Values
Standard deviation	5.664
Mean	98.066
Coffecient of variation (%)	5.775
PRESS	2045.362
R^2	0.409
Adjusted R^2	-0.181
Predicted R^2	-1.689
Adequate precision	4.000

Fig.1 Effect of various physical and nutritional variables on the production of α-L- rhamnosidase by *Clavispora lusitaniae* 84. (a) Carbon sources; (b) Nitrogen sources; (c) temperature; (d) pH; (e) Rhamnose and (f) Yeast extract

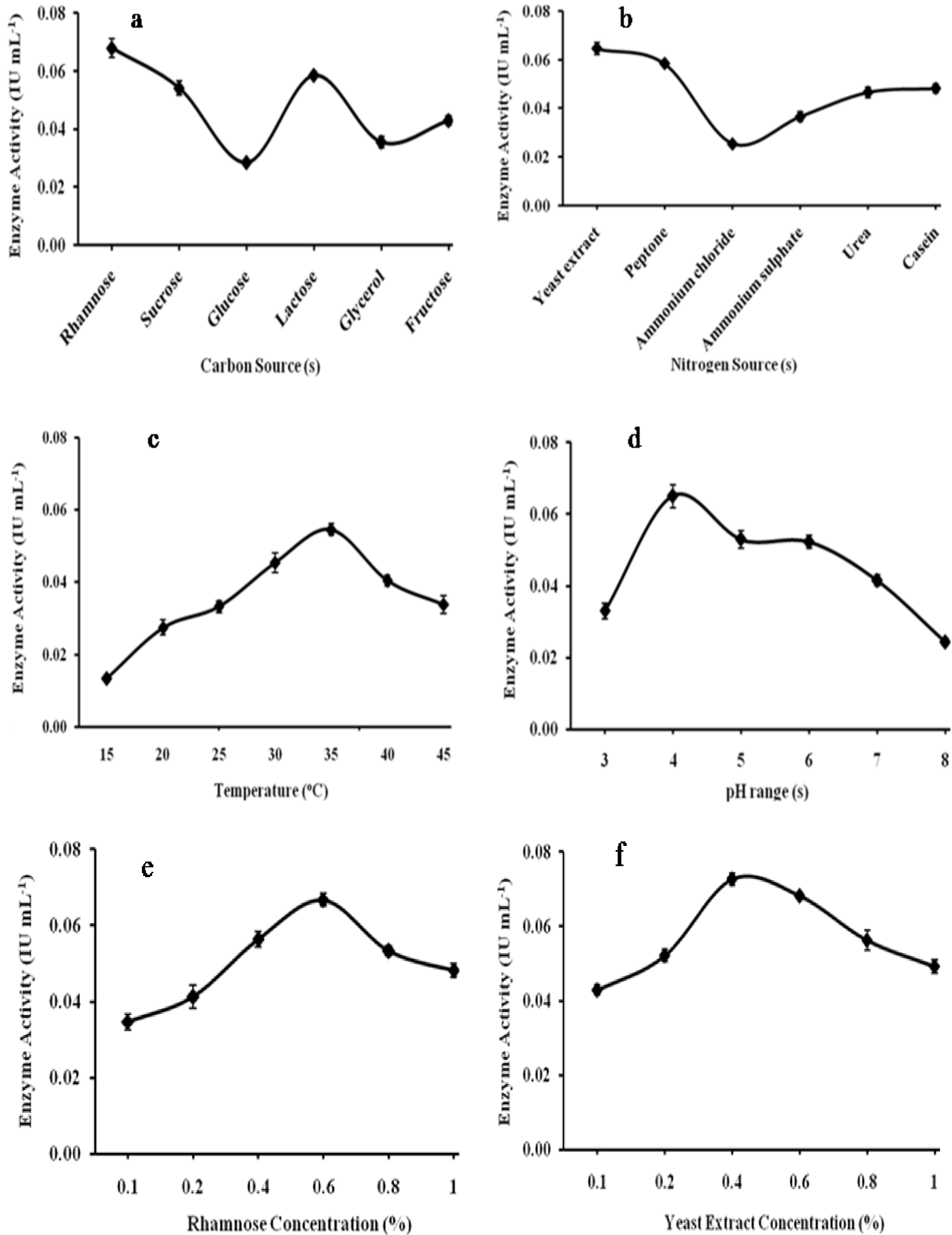
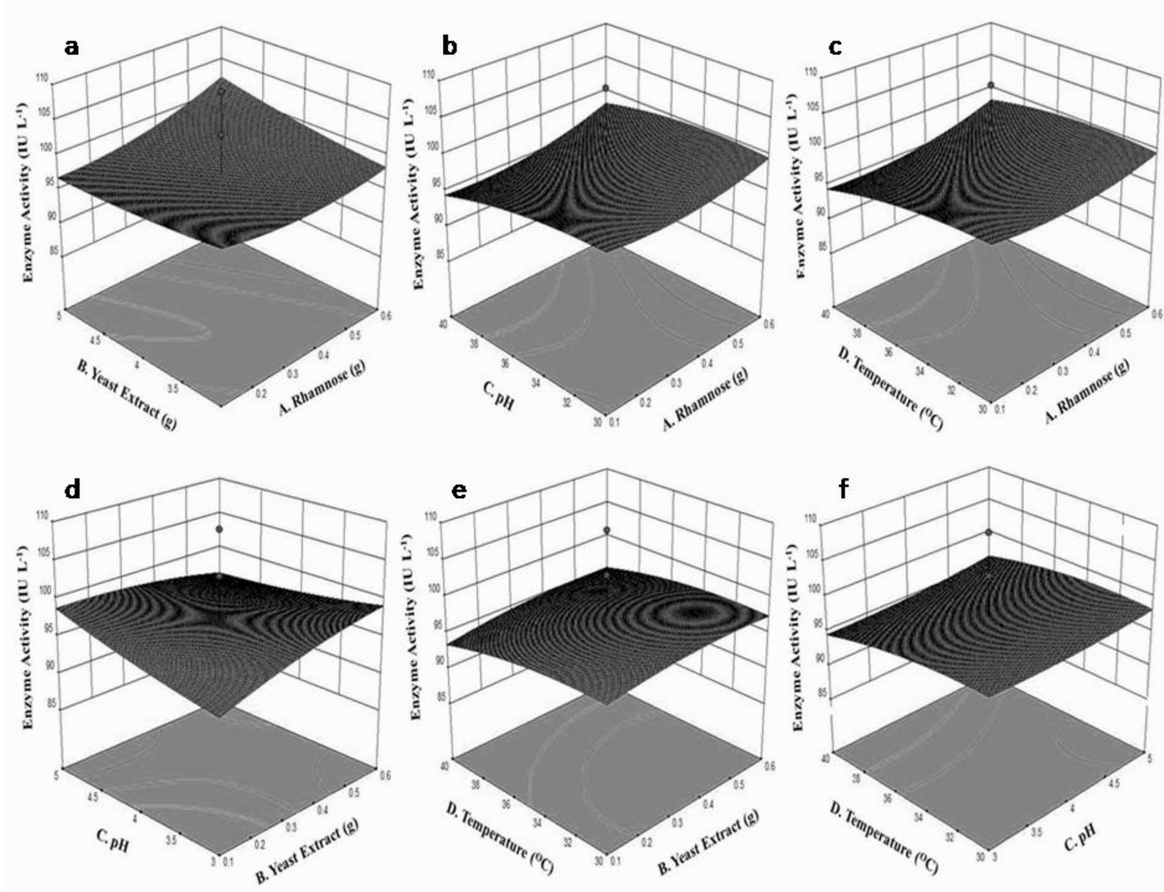


Fig.2 Three-dimensional response surface plot of α-L-rhamnosidase production by *Clavispora lusitaniae* KF633446 showing the interaction between (a) yeast extract and rhamnose; (b) pH and rhamnose; (c) temperature and rhamnose; (d) pH and yeast extract; (e) temperature and yeast extract and (f) temperature and pH on α-L-rhamnosidase production (IU L^{-1})



Optimization of screened medium components using response surface methodology

Following the screening experiments, CCD with 30 experiments was used to determine the optimal levels of the four significant factors (rhamnose, yeast extract, pH and temperature) that affected α-L-rhamnosidase production. The design of experiments and respective experimental and predicted α-L-rhamnosidase activities are given in Table 2. The results obtained after CCD were analyzed by standard analysis of variance (ANOVA), which gave the following regression equation (in terms of coded factors) of the levels of α-L-rhamnosidase produced (Y) as a function of rhamnose (A), yeast extract (B), pH (C) and temperature (D):

$$Y = 97.28 + 1.29A + 0.708B + 0.54C - 1.29D - 0.68AB - 1.4AC$$

$$Y = + 0.56AD - 1.9BC + 0.18BD + 0.31CD$$ (Equation 1)

$$Y = + 1.3A^2 - 0.69B^2 + 0.427C^2 - 0.9479D^2$$

The significance of the model was also analyzed by analysis of variance (ANOVA) for the experimental design (Table 3). Values of "$p > F$" less than 0.0500 indicate model terms are significant. In this case there are no significant model terms. Values greater than 0.1000

indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model. The model F- value of 0.69 implies the model is not significant relative to the noise. There is a 74.89% chance that a F- value this large could occur due to noise. Significant process variables were A, B, C, D, A^2, B^2, C^2, D^2, AB, AC, AD, BC, BD and CD. The "lack of fit F-value" of 0.58 implies the lack of fit is not significant relative to the pure error. There is a 77.89% chance that a "lack of fit F-value" this large could occur due to noise. The non-significant lack of fit of the tested model also indicated that the model was a good fit (Table 3).

A low value of coefficient of variation (5.77%) indicates that experimental data were precise and reliable. The goodness of fit of the model was also checked by the coefficient of determination, R^2, which was calculated to be 0.4094. This implies that 40.9479% of experimental data of the α-L-rhamnosidase activity was compatible with the data predicted by the model and only 59.06% of the total variations were not explained by the model.

The R^2 value is always between 0 and 1, and a value greater than 0.75 indicates aptness of the model. For a good statistical model, R^2 value should be close to 1.0. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The result 4.001 indicates an adequate signal and this model can be used to navigate the design space. A negative predicted R^2 (-1.689) implies that the overall mean is a better predictor of the response than the current model. The adjusted R^2 value corrects the R^2 value for the sample size and for the number of terms in the model. The value of the adjusted R^2 was -0.18. All these considerations indicate good adequacy of the regression model (Table 4).

The three-dimensional response surface and contour plots described by the regression model are presented in Figure 2. These plots were obtained from the pair wise combination of two independent variables, while keeping the other two variables at their center-point levels. From the curve of three-dimensional plots, optimal composition of medium components can be identified.

The contour plots highlight the roles played by the process variables (rhamnose, yeast extract, pH and temperature) and their interactive effects. From Fig. 2 it is evident that increase in concentration of variables had a positive influence on α-L-rhamnosidase activity until an optimum value was reached, beyond which variables had significant negative influence on the α-L-rhamnosidase activity. The contour plots show a rather broad plateau region in which the activities change relatively little when the nutrient concentrations were varied. This indicates that the optimal solution can accommodate small errors or variability in the experimental factors.

The results presented here demonstrate that among many methods to improve enzyme activity and yield, optimization of medium components and cultivation conditions remains a facile and feasible way to enhance enzyme activity as well as yield. RSM was found to be very effective in optimizing the medium components in manageable number of experimental trials.

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