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Optimization of vanillin production using isoeugenol as substrate by *Aspergillus niger* I-1472

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Abstract

Response Surface Methodology (RSM) was used in the study to optimize the production of vanillin from isoeugenol through fermentation by *Aspergillus niger* I-1472. Three factors were studied which include amount of isoeugenol, resin (Amberlite XAD-4) and Span 80. During fermentation, isoeugenol as substrate were vortexed with Span 80 and added into the culture on Day 4. Resin (Amberlite XAD-4) was added into the medium the following day. The predicted optimum medium combination consisted of 3.61 g/L of isoeugenol, 5.8% (g/mL) of Amberlite XAD-4 resin and 0.37% of Span 80 with an expected vanillin production of 0.137 g/L. Verification test showed that the model produced similar predicted and experimental values.

Introduction

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the main component of natural vanilla derived from beans of Vanilla orchid (*Vanilla planifolia* Andrews, syn. *V. fragrans* (Salisb. Ames)) (Walton *et al*., 2003). In the world market, there is a demand of more than 1200 tons of vanillin each year, but only 1% is from vanilla beans. Vanilla is widely used as flavoring in foods such as cookies, muffins, cakes, ice-creams and soft drinks; cosmetic, and pharmaceutical products.

Most of the vanillin used is produced synthetically at an estimated rate of 13000 tons annually. Synthetic vanillin is mainly produced from guaiacol which is a petrochemical product. Natural vanillin supplies only 2% of the vanillin market (Havkin-Frenkel and Belanger, 2008). Synthetic vanillin costs approximately USD $11-15 kg^{-1}. However, natural vanillin has a significantly higher price where vanillin produced from microorganism has a price of about USD $1,000 kg^{-1} (Korthou and Verpoorte, 2007; Converti *et al.*, 2010). Demand for natural vanillin has been increasing worldwide due to increasing concerns regarding health and nutrition (Ashengroph *et al*., 2011; Zhao *et al*., 2006). In addition, there is greater preference for natural vanillin due to the presence of racemic mixtures in synthetic vanillin production (Rana *et al.*, 2013).

Isoeugenol is the main component of natural essential oils of clove trees. Production of vanillin from isoeugenol biotransformation is more economical because isoeugenol costs only USD $5 kg^{-1} (Priefert *et al.*, 2001). The first known microorganisms to transform isoeugenol to vanillin is *Aspergillus niger* ATCC 9142 with only 10% efficiency, due to the degradation of produced vanillin into vanillic acid and vanilyl (Ashengroph *et al.*, 2008; Ashengroph *et al*., 2011). Lesage-Meessen *et al.* (1996) utilized *Aspergillus niger* I-1472 in a study bioconversion two step biconversion of ferulic acid to vanillin. In the first step, *A.niger* I-1472 was converted ferulic acid to vanillic acid. The second step involved the conversion of vanillic acid to vanillin by *Pycnoporus cinnabarinus*. From Srivastave *et al.* (2010), species of Aspergillus were screened for bioconversion capability from eugenol to vanillin via one step conversion. *A.niger* and *A.flavus* were found capable to convert eugenol to vanillin.

According to Stentelaire *et al.* (2000), Amberlite XAD - 2 resin was added to the culture medium of *Pycnoporus cinnabarinus* MUCL 39533 to absorb produced vanillin and prevent vanillic acid to be transformed to methoxyhydroquinone. Methoxyhydroquinone was an unwanted by-product and will result in vanillin reduction. XAD - 2 resin can also prevent toxicity of vanillin to the metabolism

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of fungi by the vanillin during fermentation and biotransformation (Zhao *et al*., 2006). Isoeugenol, having ρo/w of 3.04, is barely soluble in water (700mg - 810 mg/L) (Wangrangsimagul *et al.*, 2011). To overcome the low accessibility of isoeugenol during the biotransformation in this study, Span 80 was used as surfactant to mix with isoeugenol. Span 80 had low hydrophile-lipophile balance (HLB) value, 4.3 which is oil soluble surfactant (Santini *et al*., 2006).

Biotransformation of isoeugenol, a natural resourceful substrate, to vanillin is environmentally friendly and economically beneficial (Ashengroph *et al*., 2008). USFDA and European legislation has identified vanillin production from raw materials through biotechnology is "natural" (Hua *et al*., 2007). The objective for this study was determined the optimum combination of isoeugenol, XAD-4 resin and Span 80 for production of vanillin using A.niger I-1472.

Materials and Methods

Materials

Isoeugenol (98%, cis-trans mixture), Span 80, Amberlite resin XAD-4, Vanillic acid for synthesis, KH2PO4, HPLC grade Methanol, Ethyl acetate, maltose and potato dextrose agar (PDA) were all purchased from Merck Malaysia; vanillin (99%), yeast extract, Trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich. $CaCl_2.2H_2O$, and $MgSO_4.7H_2O$ were purchased from R & M Chemicals, Malaysia and diammonium tartrate from Fluka Chemicals, Malaysia.

Fermentation conditions

Aspergillus niger I-1472 was obtained from Institute Pasteur, France. The fungus was grown in PDA petri dish for 4 days at 30°C. A suspension of spores was obtained from washing the petri dish cultures with 10 ml of sterilized 0.1% NaCl. Then, 1 x 10^7 cfu/ml of *Aspergillus niger* I-1472 was inoculated into 100 ml basal medium (pH 5.61) in 250 ml Erlenmeyer flask. The basal medium consisted of: maltose, 20.0 g/L; diammonium tartrate, 1.842 g/L; yeast extract, 0. 5g/L; KH_2PO_4, 0.2 g/L; $CaCls.2H_2O$, 0.0132 g/L; $MgSO_4.7H2O$, 0.5 g/L (Stentelaire *et al*., 2000). Culture was incubated at 30°C in incubator shaker at 200 rpm for 9 days. Isoeugenol was added into the medium on the 4th day of fermentation during the culture's stationary phase. Span 80 (Table 1) was mixed and vortexed together with isoeugenol before being added into the medium. Resin was added (Table 1) 24 hr after the addition of isoeugenol into the culture (Hua *et al.*, 2007).

Preparation for Amberlite XAD-4 resin

The polyaromatic Amberlite resin XAD-4 from Merck (20 - 60 mesh size, 725 m^2/g, 50 Å) was used in the experiment. Before use, the resin was soaked in methanol for 24 hr at room temperature. The resin was washed thoroughly with distilled water in a Buchner funnel and oven dried at 70°C overnight. The dried resin was then wrapped with muslin cloth made into a small bag according to the weight shown in Table 1. Before the resin bags were added into the culture, the resin bags were suspended in distilled water and autoclaved for 15 min (Xu *et al.*, 2009).

Medium and Amberlite resin XAD-4 extraction after fermentation

After fermentation, the medium was filtered with Whatman 93 wet strength filter paper using a vacuum pump. The medium was mixed with ethyl acetate (1:1, v/v). The resin was eluted with ethyl acetate (1:4, w/v) for 2 hr at 30°C in an incubator shaker at 200 rpm. The amount of isoeugenol, resin and Span 80 was added as shown in Table 1. The organic layer obtained from the medium was then combined with the elution from the resin and evaporated (Buchi Rotavapor R-114) (Shimoni *et al.*, 2000; Zhao *et al*., 2006). The residue was dissolved in 10 ml of methanol and filtered for HPLC analysis.

Analytical Methods and Intrumentration

Vanillic acid, vanillin and isoeugenol were analyzed by HPLC using a Chromolith RP-18 e (100 x 4.6 mm; 2 µm particle size) with 30°C. The mobile phase with a flow rate of 1.2 mL/min consisted of a mixture of two solvents: A. 1mM trifluoroacetic acid and B. HPLC graded methanol. The elution profile used was as following: Solvent A started at 85%, holding 7 min, then decreased to 40% at 8 min till 14 min, returned to 85% at 15 min and the column was re-equilibrated for 5 min before the next injection. PDA detector was monitored at 254 nm (Li *et al.*, 2004).

Experimental design and statistical analysis

A five level, three variables central composite rotatable design (CCRD) was applied to estimate the relationship between the variables as shown in Table 1. This study involved 20 combinations of medium formulation carried out with eight factorial points, six axial points and six center points. Data were analyzed by using the Design-Expert 6.0.10. The α-level was set as 5%. Experimental data was fitted with statistical models, to produce the response

Table 1. Treatment combinations and response

Run	Variable level			Amount of vanillin (g/l) *Y*
	Isoeugenol (g/L) X_1	Resin [% (g/ml)] X_2	Span 80 [% (ml/ml)] X_3	
1	6.48 (0)	3.00 (0)	0.25 (0)	0.082
2	6.48 (0)	3.00 (0)	0.25 (0)	0.083
3	6.48 (0)	3.00 (0)	0.00 (-1.682)	0.105
4	6.48 (0)	3.00 (0)	0.25 (0)	0.090
5	3.91 (-1)	4.78 (1)	0.10 (-1)	0.075
6	10.80 (1.682)	3.00 (0)	0.25 (0)	0.078
7	3.91 (-1)	1.22 (-1)	0.10 (-1)	0.032
8	9.05 (1)	1.22 (-1)	0.40 (1)	0.043
9	6.48 (0)	3.00 (0)	0.25 (0)	0.085
10	6.48 (0)	3.00 (0)	0.25 (0)	0.096
11	9.05 (1)	4.78 (1)	0.10 (-1)	0.096
12	9.05 (1)	1.22 (-1)	0.10 (-1)	0.045
13	3.91 (-1)	4.78 (1)	0.40 (1)	0.139
14	6.48 (0)	3.00 (0)	0.25 (0)	0.097
15	9.05 (1)	4.78 (1)	0.40 (1)	0.102
16	2.16 (-1.682)	3.00 (0)	0.25 (0)	0.077
17	3.91 (-1)	1.22 (-1)	0.40 (1)	0.023
18	6.48 (0)	0.00 (-1.682)	0.25 (0)	0.026
19	6.48 (0)	6.00 (1.682)	0.25 (0)	0.135
20	6.48 (0)	3.00 (0)	0.50 (-1.682)	0.066

X_1: Amount of isoeguenol; X_2: Amount of Amberlite resin XAD-4; X_3: Amount of SPAN 80

surface. Models were considered suitable when it is significant based on ANOVA, insignificant lack-of-fit test and R^2 more than 0.75. The chosen models were subsequently optimized based on the optimization criteria of minimum of isoeugenol, while others are in range. Validation method used between the predicted and experimental value was Root Mean Squared Deviation (RMSD) (Gauch *et al*., 2003).

Result and Discussion

Developing a regression model

From the *A.niger* I-1472 fermentation, vanillin produced as response was analyzed using Design-Expert 6.0.10 software. Result of the experiment is as shown in Table 1. Statistical analysis of the model and the equation for vanillin produced by *A.niger* fermentation is presented in Table 2. The model was significant ($p<0.05$) with insignificant lack of fit ($p>0.05$) and adjusted regression coefficient (R^2) of 0.858 suggesting the model adequately fits the experiment data (Ghosh *et al*., 2012).

Coefficient analysis

Table 3 model coefficients for vanillin produced by the fermentation of *A.niger,* b_1 (amount of isoeugenol) did not show any significant linear effect on the amount of vanillin. Similarly, no significant quadratic and cubic effects of amount of iseougenol was observed. This may due to the inhibiting effect of iseoguenol on vanillin producing cultures. Shimoni *et al.* (2000) reported that isoeugenol at a concentration

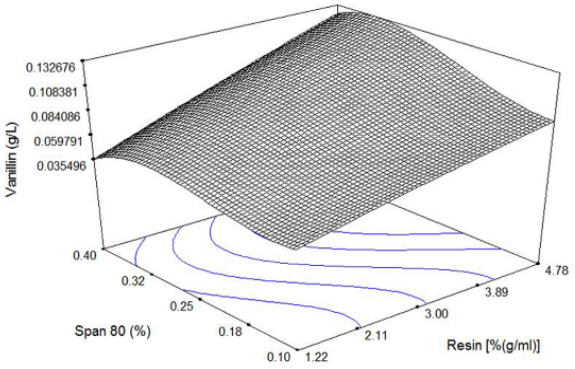

Figure 1. 3D response surface showing effect of resin, X_2 [% (g/mL)] and Span 80, X_3(%) to vanillin (g/L) produced by *A.niger* I-1472

as low as 0.1% (w/v) could inhibit the growth of vanillin-producing *B. subtilis* B2. In addition, Kasana *et al.* (2007) reported that isoeugenol with concentration higher than 1% significantly reduced the vanillin production of *P. chlororaphis* CDAE5.

From Table 3 model coefficients for amount of resin (b_2) and amount of Span 80 (b_3) were significant ($p<0.05$). The linear coefficients for b_2, b_3 showed a positive effect which indicated that vanillin produced increased 104.55 % with increasing amount of resin from 3 to 6% and vanillin produced increased 68.89% with increasing (from 0.25 to 0.4%) amount of Span 80. Hua *et al*. (2007) reported that adding adsorbent resin DM11, reduced the chemical toxicity and product repression and thus enhancing the vanillin production to 19.2 g/L with a 54.5% total molar yield. According to Zheng *et al.* (2007), when 10 g of

Table 2. Statistical analysis of the model for the vanillin produced through *A.niger* fermentation

	Equation	Model significance	Lack-of-fit	R^2
Coded	$Y = 0.085 + 3.975 \times 10^{-3} x_1 + 0.034 x_2 + 0.030 x_3 - 1.903 \times 10^{-3} x_1^2 - 1.750 \times 10^{-3} x_2^2 - 0.012 x_3^2 - 5.678 \times 10^{-3} x_1x_2 - 5.870 \times 10^{-3} x_1x_3 + 0.010 x_2x_3 + 8.742 \times 10^{-3} x_1^3 - 0.021 x_3^3$	0.001	0.164	0.858
Actual	$Vanillin = 0.012 + 6.849 \times 10^{-3} X_1 + 0.021 X_2 - 0.761 X_3 + 8.331 \times 10^{-4} X_1^2 - 5.500 \times 10^{-4} X_2^2 + 4.327 X_3^2 - 1.338 \times 10^{-3} X_1X_2 - 0.017 X_1X_3 + 0.038 X_2X_3 - 6.497 \times 10^{-5} X_1^3 - 6.492 X_3^3$			

Y = production of vanillin at the end of fermentation in g/L.
X_1 = Amount of isoeugenol (g/L)
X_2 = Amount of Amberlite resin XAD-4 [% (g/mL)]
X_3 = Amount of Span 80 [% total (v/v)]

Table 3. Coeficient estimates and p value in the regression model selected through variable selection

Model coefficient	Coefficient estimate	*P* value [1]
b_o	0.085	0.0001
b_1	3.957×10^{-3}	0.5595
b_2	0.034	<0.0001
b_3	0.030	0.0145
b_{11}	-1.903×10^{-3}	0.5962
b_{22}	-1.750×10^{-3}	0.6255
b_{33}	-0.012	0.0917
b_{12}	-5.678×10^{-3}	0.1927
b_{13}	-5.870×10^{-3}	0.1796
b_{23}	0.010	0.0465
b_{111}	8.742×10^{-3}	0.7716
b_{333}	-0.021	0.0189

HZ802 resin was added, vanillin adsorbed increased and residual in the media decreased. During vanillin production from isoeugenol using *Bacilllus fusiformis*, 50 g/l isoeugenol and 12.5 g resin HB-8 in 20 ml medium culture produced 8.10 g/L of vanillin under optimum condition. However, further increasing in the amount of resin HD-8 did not increase the yield of vanillin (Zhao *et al*., 2006). Shimoni *et al.* (2000) showed the growth characteristic of *B.subtilis* strain B2 on isoeugenol had improved with 10% (w/w) of resin Amberlite XAD-2 added during the fermentation. Stentelaire *et al*. (2000) reported that 100 g/L of resin Amberlite XAD-2 was used during P.cinnabarinus fermentation had increased vanillin production to 1575 mg/L and reduced the amount of methoxyhydroquinone. The XAD-2 resin trapped the vanillin produced and reduced the degradation of vanillic acid into methoxyhydroquinone thus preventing vanillin toxicity to fungal metabolism. Figure 1 shows the response surface and the contour plot show the conditions for vanillin produced by the interaction between the amount of resin and Span 80. It was observed that production of vanillin increase with the increasing of resin at lower concentration of Span 80. The vanillin content increased from 0.11 g/L to 0.13 g/L with the increase of Span 80 from 0.32% until the optimum point level of 0.40% and subsequently decreased after the optimum point.

The commonly used emulsifier is in the range of 0.1 to 1 % of the composition or preferably from 0.1 to 0.5% by weight of the composition (Jones, 2012). From Lee *et al.* (2011), surfactants were used during the fermentation of *E. coli* to produce retinal. Span 80 was the most effective surfactant in increasing cell mass, retinal production and specific retinal content. Surfactant is lowering the surface tension between liquid and oil and will increase cell wall permeability and improve substrate uptake and product formation.

Optimimzation of vanillin production

The predicted optimum value for the factors were: Isoeugenol - 3.61 g/L; Resin – 5.80 g/ml and SPAN 80 – 0.37% with a predicted response value of 0.161 g/L vanillin. The optimum point was validated by comparing between the optimum-point and center-point. From the experimental result, the optimum point response was 0.148 g/L ± 0.013. From the RMSD method, there is a 0.023 difference between experimental and calculated results. Center-point obtained from the RSM was Isoeugenol = 6.48 g/l; Resin = 3.00 g/ml; SPAN 80 = 0.25% with calculated = 0.085 g/L vanillin production. Experimental result for central point was 0.089 g/L ± 0.017 with 0.017 difference. Small RMSD value indicates that the validity of the obtained values compare with the expected values. Both the optimum-point and center-point experiment response were within the estimated range. The amount of vanillin produced in this system was higher compared to the study by Abraham *et al.* (1988) which produced 0.08 g/L of vanillin using *Aspergillus niger* ATCC 9142. However, the amount of vanillin produced in this study was lower compared to the study by Zhao *et al*. (2006) which produced 8.1 g/L vanillin using *Bacillus fusiformis* CGMCC1347.

Conclusion

Model was adequately fitted to the response of vanillin produced. The predicted optimization condition from the RSM is: Isoeugenol - 3.61 g/L; Resin – 5.80 g/ml and SPAN 80 – 0.37% with a calculated value of 0.161 g/L vanillin production.

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