การศึกษาองค์ประกอบของอาหารเลี้ยงเชื้อที่เหมาะสมสำหรับการผลิตเอนไซม์ตกตะกอนน้ำนม โดยเชื้อรา A26 จากการหมักรำข้าวโดยกระบวนการหมักแบบแห้ง

Optimization of medium components for the production of milk clotting enzyme by a filamentous fungus A26 in solid state fermentation using rice bran

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บทคัดย่อ

จากการศึกษาการผลิตเอนไซม์ตกตะกอนน้ำนมของเชื้อรา A26 ด้วยการหมักรำข้าวในกระบวนการหมักแบบแห้ง โดยศึกษาผลของสารแหล่งไนโตรเจนชนิดต่างๆต่อกิจกรรมการตกตะกอนน้ำนม (milk clotting activity, MCA) และค่าจำเพาะของกิจกรรมการตกตะกอนน้ำนมต่อมิลลิกรัมโปรตีน (specific activity of milk clotting/mg protein, SPA) วางแผนการทดลองแบบสุ่มตลอด พบว่า การเติมแอมโมเนียมซัลเฟตให้มีปริมาณไนโตรเจนร้อยละ 0.05 นั้นให้ค่า MCA และ SPA สูงสุดที่ 1,467 หน่วยต่อมิลลิลิตร และ 3,861 หน่วยต่อมิลลิกรัมโปรตีน ตามลำดับ ส่วนการศึกษาความเข้มข้นของกลูโคส แลคโตส และแอมโมเนียมซัลเฟตต่อค่า MCA ด้วย response surface methodology สมการที่ได้ทำนายว่าในช่วงความเข้มข้นที่ศึกษา การเติมกลูโคสและแลคโตสอัตรา 0.040 กรัมต่อกรัมรำข้าว ร่วมกับแอมโมเนียมซัลเฟตอัตรา 0.004 กรัมต่อกรัมรำข้าวให้ค่า MCA สูงสุดที่ 1,727 หน่วยต่อมิลลิลิตร

Abstract

Microbial production of milk clotting enzyme by an airborne filamentous fungus A26 was studied in solid state fermentation using rice bran as a substrate. Effects of various nitrogen sources on milk clotting activity (MCA) and specific activity of milk clotting/mg protein (SPA) were investigated using a completely

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randomized design. The highest levels of MCA (1,467 S.U./ml) and SPA (3,861 S.U./mg protein) were obtained when ammonium sulfate was supplied to provide 0.05% nitrogen. Impacts of glucose, lactose, and ammonium sulfate on MCA were evaluated by response surface methodology. The results suggested that glucose, lactose, and ammonium sulfate enhanced MCA. Within the ranges of concentration studied, the highest MCA at 1,727 S.U./ml was predicted by the model when glucose, lactose, and ammonium sulfate were supplied at 0.040, 0.040, and 0.004 g/g rice bran, respectively.

คำสำคัญ: เอนไซม์ตกตะกอนน้ำนม รำข้าว กระบวนการหมักแบบแห้ง
Keywords: milk clotting enzyme, rice bran, solid state fermentation

Introduction

Rennet is an aspartate protease enzyme used in cheese making (Kumar et al., 2005). The coagulation of milk casein by rennin takes place in two phases, the enzymatic and non-enzymatic phases. In the enzymatic phase, casein is hydrolyzed by rennin to form para-casein which subsequently becomes a curd in the non-enzymatic phase under appropriate temperature and concentration of calcium ions (Fox et al., 2000). Calf rennet is obtained from the stomach of unweaned calves (Dutt et al., 2009). Previously, calf rennet was extensively used in cheese industries. Nevertheless, due to the increasing of global cheese demand in the last few decades and a limited availability of calf rennet, the price of commercial calf rennet greatly rises up (Seker et al., 1999). Therefore, alternative milk clotting enzymes from other sources are necessary for cheese manufacturers (Lopes et al., 1998). Microorganisms generally recognized as efficient milk-clotting enzyme producers are such as *Mucor miehei*, *Mucor pusillus* (Seker et al., 1999), *Aspergillus oryzae*, and *Endothia parasitica* (Silveira et al., 2005). A number of researches have been accomplished on an investigation of calf rennet substituted enzymes from microorganisms (Hashem, 1999; Hashem, 2000). However, fermentation conditions as well as medium components reported for the rennet production by those organisms are widely varied.

The current research was undertaken to investigate the effect of various nitrogen sources supplementation on the production of milk clotting enzyme by a filamentous fungus A26. The fungus was chosen since it exhibited a high MCA and SPA and a low proteolytic activity in our preliminary study (data not shown). The fermentation was carried out in a solid state fermentation using rice bran as a substrate. Then, the subsequent experiment was run using response surface methodology to evaluate the effect of glucose, lactose, and ammonium sulfate on MCA.

Materials and Methods

Microorganism

An airborne filamentous fungus, referred to as A26, was isolated in our laboratory. Working culture was maintained on potato dextrose agar (HIMEDIA, India) at 4°C with periodic subculturing. Stock culture was stored in 15% glycerol at -80°C, using 4-5 pieces of PDA culture per vial.

Fermentation medium and culture conditions

Solid state fermentation was carried out in 250 ml Erlenmeyer flasks using rice bran as a substrate. Unless otherwise mentioned, the fermentation medium contained 10 g rice bran (purchased from the local store), 2 g casein (Sigma-Aldrich, Singapore), and 10 ml distilled water, pH 6.0 ± 0.2. The inoculum was prepared by cultivating the fungus A26 on a PDA slant at 30°C for 5 d. Then, 10 ml of sterile distilled water was added and the culture was scraped from the agar surface. Subsequently, the culture suspension was aseptically transferred to sterilized fermentation medium and the fermentation was allowed at 30°C for 10 d. Since the fungus A26 did not sporulate on PDA, its mycelia were used as inoculum in place of spores.

To extract the enzyme from the medium, 20 ml of sterile distilled water was added and stirred for 30 min. Then, the crude enzyme was filtered through a sterile Whatman No.1 filter paper and the filtrate was collected.

Analytical methods

MCA was determined according to the method described by Arima et al. (1968, 1970). The MCA was reported in terms of Soxhlet Units (S.U.) where the amount of enzyme which clots the skim milk in 1 min under specified conditions was defined to contain 400 S.U. of activity. Protein determination was made following the method of Bradford (1976). Specific activity of milk clotting/mg protein (SPA) was calculated by diving MCA by the protein concentration.

Effects of various nitrogen sources on MCA and SPA

A comparative study of different nitrogen source supplementation was conducted in triplicate. Peptone (Sigma-Aldrich, Singapore), yeast extract (Scharlau, Singapore), corn steep liquor (Lab System, Thailand), casein acid hydrolysate (Difco, USA.) and ammonium sulfate (Sigma-Aldrich, Singapore) were separately added into fermentation medium, each at the amount that contributed 0.05% elemental nitrogen. The fermentation was accomplished at 30°C for 10 d.

Effects of glucose, lactose and ammonium sulfate on MCA, evaluated by RSM

The Box-Behnken design was used to investigate the effects of glucose, lactose and ammonium sulfate on MCA of the fungus A26 by RSM. The independent variables of the experiments were coded for statistical purposes as follow:

$$X_1 \text{ (glucose)} = \frac{glucose - 0.02}{0.02} \quad (1)$$

$$X_2 \text{ (lactose)} = \frac{lactose - 0.02}{0.02} \quad (2)$$

$$X_3 \text{ (ammonium sulfate)} = \frac{ammonium\ sulfate - 0.002}{0.002} \quad (3)$$

The relationships and interrelationships of the variables were determined according to the following quadratic equation:

$$Y = b_0 + \Sigma b_i x_i + \Sigma b_{ii} x_i^2 + \Sigma b_{ij} x_i x_j \quad (4)$$

Where Y is the predicted response of dependent variable, MCA; b_0, b_i, $b_{ii,}$ b_{ij} are regression coefficients which express the constant, the linear effect, the squared effect, and the interaction effect, respectively (i, j = 1-3); x_i and x_j are the independent variables (i, j = 1-3). Actual values of the coded independent variables ($x_{1,} x_{2,} x_3$) used in Box-Behnken design were described as follow.

Variables (g/g rice bran)	Designate	Range and levels		
		-1	0	1
Glucose	x_1	0.000	0.020	0.040
Lactose	x_2	0.000	0.020	0.040
Ammonium sulfate	x_3	0.000	0.002	0.004

Results and Discussion

Nitrogen sources have been reported to influence MCA of microorganisms. Hashem (1999) reported that the combination of yeast extract and peptone or baker's yeast alone were suitable for milk clotting enzyme productivity of *Penicillium oxalicum*. Silveira et al. (2005) stated that the addition of corn steep liquor at 4.4 g/l significantly improved MCA. In this work, effects of peptone, yeast extract, corn steep liquor, casein acid hydrolysate, and ammonium sulfate on MCA and SPA were investigated and the results were revealed in Table 1.

From the results, all nitrogen sources, except corn steep liquor, improved MCA of the fungus A26. Maximum enzyme activity and specific activity of MCA per mg protein were observed when ammonium sulfate was supplied. It was interesting that ammonium sulfate which is an inorganic nitrogen provided better MCA than the organic nitrogen tested although the organic nitrogen contained various vitamins and growth factors. This possibly was because the vitamins and growth factors required were already available in rice bran and casein, which were the major components in the fermentation medium. Another advantage of ammonium sulfate is that it is more cost-effective, comparing to peptone, yeast extract, and casein acid hydrolysate.

Effect of glucose, lactose and ammonium sulfate on MCA, evaluated by RSM

From the results obtained in Table 1, the addition of ammonium sulfate at the amount that provided 0.05% elemental nitrogen appreciably promoted the production of milk clotting enzyme by the fungus A26. Therefore, ammonium sulfate was used in this study along with glucose and lactose to determine the effects of each parameter and their interaction effects on MCA by RSM. Glucose was introduced in this study since several research works reported the enhancement of glucose on MCA when the appropriate levels were utilized (Silveira et al., 2005; De Lima et al., 2008; Beyenal et al., 1999). Lactose was studied since it's a milk component.

Table 1. Effects of peptone, yeast extract, corn steep liquor, casein acid hydrolysate, and ammonium sulfate on MCA and SPA

Nitrogen sources[a]	MCA (S.U./ml)	Protein (mg/ml)	SPA (S.U./mg protein)
Control[b]	733	0.27	2,715
Peptone	800	0.48	1,667
Yeast extract	1,040	0.49	2,122
Corn steep liquor	724	0.47	1,540
Casein acid hydrolysate	1,120	0.43	2,605
Ammonium sulfate	1,467	0.38	3,861

[a]Nitrogen sources were separately added into fermentation medium, each at the amount that contribute 0.05% elemental nitrogen.
[b]Control is the fermentation medium without additional nitrogen supplementation.

Using the Box-Behnken method, a total of fifteen experiments with various combinations of glucose, lactose and ammonium sulfate levels were carried out. MCA was analyzed and the result was presented in Table 2. Then, the multiple regression analysis was applied and the regression coefficients, t-value, and probability value (P-value) were indicated in Table 3. According to Box and Draper (2007), Myers and Montgomery (2002), the P-value indicated the significance of each variable coefficient. From the multiple regression analysis (Table 3), the P-value of the linear terms of glucose and lactose and the squared terms of glucose and ammonium sulfate were significant ($p<0.05$), indicating that glucose, lactose, and ammonium sulfate affected MCA. Thus, coefficients of those variables were included in the quadratic equation. However, the coefficients of all interaction terms ($x_i x_j$) were dropped off since their P-values were higher than 0.05, interpreting that the interaction effects of glucose, lactose, and ammonium sulfate were insignificant. The quadratic equation was then generated as follow.

$$MCA = 1111.00 + 82.25x_1 + 267.75x_2 + 97.00x_1^2 + 168.75x_3^2 \quad (5)$$

Table 2. Box-Behnken design and the results of MCA

Run	Glucose* (x_1)	Lactose* (x_2)	Ammonium sulfate* (x_3)	MCA (S.U./ml)
1	-1	-1	0	824
2	1	-1	0	996
3	-1	1	0	1,448
4	1	1	0	1,524
5	-1	0	-1	1,257
6	1	0	1	1,600
7	-1	0	-1	1,203
8	1	0	1	1,447
9	0	-1	-1	996
10	0	1	1	1,600
11	0	-1	-1	960
12	0	1	1	1,523
13	0	0	0	1,111
14	0	0	0	1,111
15	0	0	0	1,111

*Values of glucose, lactose, and ammonium sulfate presented were coded.

Table 3. Regression coefficients, t-value, and P-value for MCA using Box-Behnken design and analyzed by RSM

Variables	Coefficients	Std. error coefficient	t-value	P-value
Constant	1,111.00	33.34	33.328	0.000
Glucose	82.25	25.00	3.290	0.013
Lactose	267.75	25.00	10.709	0.000
ammonium sulfate	44.25	28.87	1.533	0.169
glucose* glucose	97.00	30.05	3.228	0.014
lactose*lactose	-10.00	30.05	-0.333	0.749
ammonium sulfate*ammonium sulfate	168.75	30.05	5.616	0.001
glucose*lactose	-24.00	28.87	-0.831	0.433

Where MCA was the predicted milk clotting activity, x_1, x_2 and x_3 were the coded values of glucose, lactose, and ammonium sulfate, respectively. The goodness of fit of the model was determined by the R^2 value. In this case, the high R^2 value (0.961) was yielded (Figure 2), demonstrating a good agreement between experimental and predicted MCA and only 0.039% of the total variation cannot be explained by the model.

From the model generated, MCA of the crude enzyme produced by the fungus A26 at different levels of glucose, lactose, and ammonium sulfate were predicted and the three-dimensional surface plot was shown in Figure 1. It was evident that glucose, lactose, and ammonium sulfate enhanced the efficiency of the fungus A26 on the production of milk clotting enzyme. Within the concentration ranges studied, the model predicted the highest MCA at 1,727 S.U./ml when glucose, lactose, and ammonium sulfate were supplied at 0.04, 0.04, and 0.004 g/g rice bran, respectively. The correlation between predicted and experimental values of MCA was shown in Figure 2.

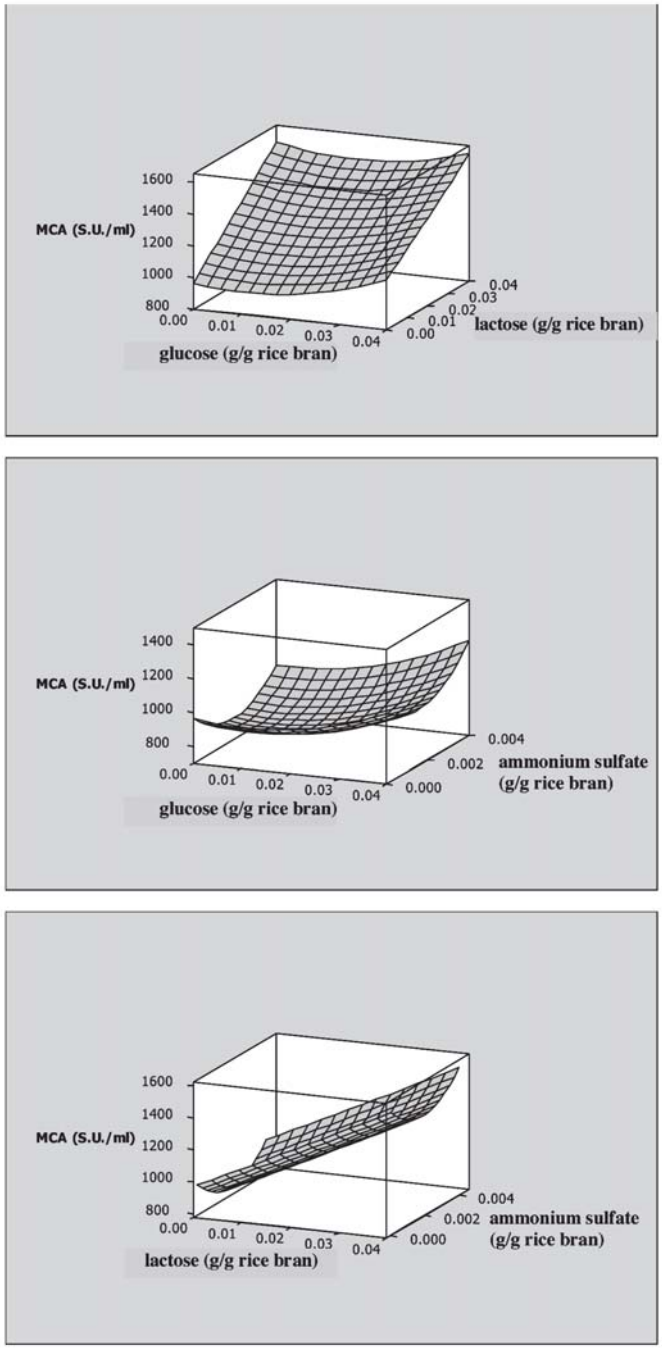

Figure 1. Response surface curve of predicted MCA by the fungus A26 when the levels of glucose, lactose, and ammonium sulfate were varied. (A) Ammonium sulfate was held at 0.002 g/g rice bran. (B) Lactose was held at 0.02 g/g rice bran. (C) Glucose was held at 0.02 g/g rice bran.

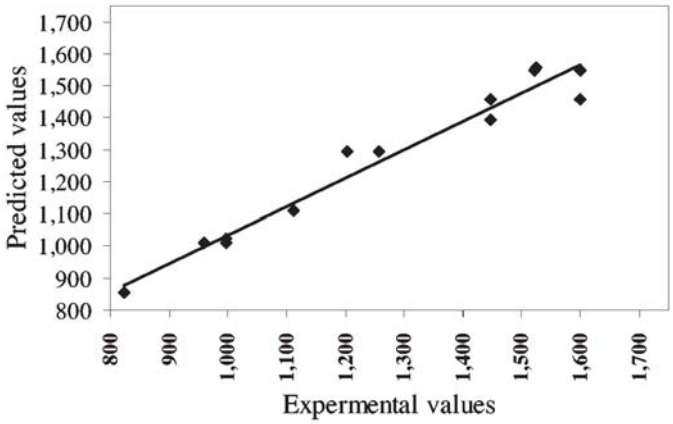

Figure 2. Experimental vs. predicted values plot for MCA by the fungus A26 when the levels of glucose and lactose were varied in the range of 0.00-0.04 g/g rice bran while ammonium sulfate concentration was varied between 0.000 and 0.004 g/g rice bran. The R^2 value was 0.961.

Effects of the linear terms, squared terms, and interaction terms were summarized in the analysis of variance for MCA in Table 4. The small P-value of the squared term (P = 0.003) indicated the curvature in the response surface. On the other hand, the interaction effect was insignificance since P-value was higher than 0.05.

Table 4. Analysis of variance for MCA

Source	*df*	Seq SS	Adj SS	Adj MS	F-value	P-value
Regression	7	904,010	904,010	129,144	38.74	0.000
Linear	3	767,206	767,205	255,735	76.71	0.000
Square	3	134,500	134,500	44,833	13.45	0.003
Interaction	1	2,304	2,304	2,304	0.69	0.433
Residual Error	7	23,336	23,336	3,334		
Lack-of-Fit	1	6,561	6,561	6,561	2.35	0.176
Pure Error	6	16,775	16,775	2,796		
Total	14	927,346				

df = degree of freedom, Seq SS = sum of squares, Adj SS = adjusted sum of squares, Adj MS = adjusted mean squares, F-value = variance ratio, P-value = probability

Conclusion

Ammonium sulfate is a good nitrogen source for the production of milk clotting enzyme by the fungus A26 in a solid state fermentation using rice bran. The addition of glucose, lactose, and ammonium sulfate at 0.04, 0.04, and 0.004 g/g rice bran, respectively, significantly promoted the MCA of the fungus A26.

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