การหาสภาวะที่เหมาะสมในการผลิตกรดแลคติกจากแป้งมันสำปะหลังที่ผ่านการย่อยแล้ว โดย *Lactobacillus casei* TISTR 453

Optimization of lactic acid production from tapioca starch hydrolysate by *Lactobacillus casei* TISTR 453

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บทคัดย่อ

งานวิจัยนี้ได้ศึกษาอิทธิพลของแคลเซียมคาร์บอเนต สารสกัดจากยีสต์ และแมงกานีสซัลเฟต ในการผลิตกรดแลคติกจากแป้งมันสำปะหลังที่ผ่านการย่อยแล้ว หมักด้วย *Lactobacillus casei* TISTR 453 ที่อุณหภูมิ 37 องศาเซลเซียส สนาน 72 ชั่วโมง โดยให้มีน้ำตาลรีดิวซ์เริ่มต้นที่ 50 กรัมต่อลิตร จากการแปรผันอัตราแคลเซียมคาร์บอเนตระหว่าง 0-100 กรัมต่อลิตร พบว่า ค่าที่เหมาะสมคือ 50 กรัมต่อลิตร ซึ่งให้กรดแลคติกได้มากถึง 31.15 กรัมต่อลิตร และมีค่าสัมประสิทธิ์ผลผลิตกรดแลคติก ($Y_{L/S}$) สูงสุดที่ 0.73 กรัมต่อกรัมซับสเตรต ส่วนการศึกษาอัตราที่เหมาะสมของสารสกัดจากยีสต์และแมงกานีสซัลเฟตโดย response surface methodology (RSM) นั้น สมการที่ได้ทำนายว่า การใช้สารสกัดจากยีสต์และแมงกานีสซัลเฟตโมโนไฮเดรตในอัตรา 20 และ 0.05 กรัมต่อลิตรตามลำดับ จะทำให้ได้กรดแลคติก ที่ความเข้มข้นสูงสุด 42.39 กรัมต่อลิตร และได้ค่า $Y_{L/S}$ สูงสุดที่ 0.84 กรัมต่อกรัมซับสเตรต

Abstract

The influences of calcium carbonate, yeast extract, and manganese sulfate on the production of lactic acid from tapioca starch hydrolysate by *Lactobacillus casei* TISTR 453 were investigated. All experiments were carried out in a submerged fermentation for 72 h at 37°C with an initial level of reducing sugar at 50 g/l. The amount of calcium carbonate was varied between 0 and 100 g/l in the first experiment and the results indicated that 50 g/l calcium carbonate was optimal. Under the optimal condition, a high lactic acid concentration (31.15 g/l) and the highest lactic acid yield coefficient ($Y_{L/S}$) at 0.73 g lactic acid/g substrate utilized were obtained.

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To evaluate the effects of yeast extract and manganese sulfate on lactic acid production, response surface methodology (RSM) was employed. The model obtained from RSM predicted a maximum lactic acid concentration (Y) and $Y_{L/S}$ at 42.39 g/l and 0.84 g/g substrate utilized, respectively, using 20 g/l yeast extract and 0.05 g/l manganese sulfate monohydrate.

คำสำคัญ: กรดแลคติก *Lactobacillus casei,* response surface methodology
Keywords: lactic acid, *Lactobacillus casei,* response surface methodology

Introduction

Lactic acid is one of the earliest organic acids known. It was first discovered in 1780 by Scheele. Nowadays, the annual demand for lactic acid consumption has been estimated between 130,000 and 150,000 metric tons worldwide with the increasing trend annually since lactic acid is used extensively in many industries (Wee et al., 2006). Food, chemical, pharmaceutical and cosmetic industries are the four major categories of lactic acid uses. Among those, the applications in food and food-related industries, as an acidulent and food preservative, have played the most important role (Singleton and Sainsbury, 1988). Recently, lactic acid is of great interest as a monomer for the manufacturing of polylactide, which can be applied in biodegradable, biocompatible plastics and coatings (Narayanan et al., 2004). Although the production of lactic acid is possible via chemical synthesis and biotechnology, current tendency of a commercial scale lactic acid production relies more on microbial fermentation of various renewable resources since it causes less environmental pollution. Another major advantage of fermentative lactic acid production is that stereo-specific acid can be achieved by a selection of appropriate microbial strains whereas chemical synthesis yields a racemic mixture form (Hofvendahl and Hahn-Hägerdal, 2000; Narayanan et al., 2004). In addition, due to a limitation of petrochemical supplies, chemical synthesis of lactic acid becomes less interesting (Wee et al., 2006).

For decades, a lot of efforts have been put to attain efficient and cost-effective strategies for lactic acid fermentation. Utilization of low-cost agricultural products in place of refined sugars (i.e. glucose, lactose, etc) leads to a more economic and environmental friendly process. Tapioca starch is one of the most interesting resources for lactic acid fermentation as it is readily available throughout the year at a low cost. An enzymatic hydrolysis of tapioca starch provides appreciable amounts of fermentable sugars, mainly glucose, which are sufficient for a high yield lactic acid conversion (Hofvendahl and Hahn-Hägerdal, 2000). Although several reports on a direct conversion of cassava starch to lactic acid by lactic acid bacteria have been published, lactic acid yields were relatively low (Ohkouchi and Inoue, 2006; Wee et al., 2006; Xiaodong et al., 1997). This possibly is because limited species of high efficiency lactic acid producers possess amylolytic enzyme systems. In our current research, tapioca starch hydrolysate was chosen as a substrate for lactic acid fermentation by *Lactobacillus casei* TISTR 453 due to many advantages discussed earlier. *L. casei* TISTR 453 was selected as a starter culture since it yielded satisfactory amounts of lactic acid from tapioca starch hydrolysate in our screening steps (data not shown).

Media compositions always influence lactic acid formation significantly (Xiaodong et al., 1997). In this work, effects of calcium carbonate ($CaCO_3$), yeast extract, and manganese sulfate on lactic acid production were evaluated. $CaCO_3$ is a pH adjusting substance commonly introduced in organic acid fermentation media to retain the optimum pH range for starter cultures (Stanbury et al., 2003). Without an acid neutralizer, the pH of fermentation broth decreases rapidly during lactic acid fermentation due to the accumulation of acids. This leads to the cessation of microbial growth and acid production (Kotzamanidis et al., 2002). In general, optimum pH for lactic acid production by lactic acid bacteria is in the range of 5.0-7.0 (Hofvendahl and Hahn-Hägerdal, 2000). Influences of yeast extract and $MnSO_4.H_2O$ on lactic acid production were investigated by response surface methodology (RSM). Yeast extract has long been reported to be a good nitrogen source for lactic acid fermentation since it is rich in amino acids, vitamin B groups, and other growth factors required by lactic acid bacteria (Fitzpatrick et al., 2001). Growth and acid production of *L. casei* and some other lactic acid bacteria are stimulated by manganese supplementation at trace amounts as well because manganese acts as a cofactor of lactate dehydrogenase (Narayanan et al., 2004). In this study, appropriate concentrations of $CaCO_3$, yeast extract and $MnSO_4.H_2O$ were determined.

Materials and Methods

Microorganism

L. casei TISTR 453, a homofermentative lactic acid bacterium, was obtained from the Bangkok MIRCEN, Thailand. The strain was maintained in de Man Rogosa and Sharpe (MRS) broth (HIMEDIA, India) with 15% glycerol at -80°C.

Starch hydrolysis

Tapioca starch was purchased from the local store. Starch hydrolysis process was conducted in a 2 L Erlenmeyer flask containing 300 g dry tapioca starch and 1 L of 0.1M acetate buffer pH 6.0 with 0.05% $CaCl_2$. The process was carried out following the enzyme manufacturer instructions (Novo Nordisk A/S, Denmark). Liquefaction was initiated by adding 0.5 ml Bacterial Amylase Novo 240 L (BAN 240 L, Novo Nordisk A/S, Denmark), an alpha-amylase produced by *Bacillus subtilis.* The temperature was held at 70°C for 1 h. Subsequently, the pH level was adjusted to 4.5 by 10% H_2SO_4 and temperature was decreased to 60°C prior to an addition of 1.0 ml Amyloglucosidase Novo 150 L (AMG 150 L, Novo Nordisk A/S, Denmark). At this stage, the saccharification was started. After 48 h, the starch hydrolysate was heated at 100°C for 5 min to inactivate the enzyme. Starch residues were eliminated by refrigerated centrifugation at 5,000xg for 15 min. The starch hydrolysate was stored at 4°C until use.

Cultivation

Seed culture was prepared by cultivating *L. casei* TISTR 453 in a 125 ml Erlenmeyer flask containing 100 ml MRS broth and incubating for 24 h at 37°C. A 10% (v/v) inoculum was transferred to another 125 ml Erlenmeyer flask with 90 ml fermentation medium (modified MRS medium) and lactic acid fermentation was carried out. Unless otherwise stated, fermentation medium contained: 10 g proteose peptone (Sigma-Aldrich, Singapore), 10 g beef extract (Difco, USA.), 5 g yeast extract (Difco, USA), 1 ml Tween 80 (BDH, USA), 2 g $(NH_4)_3C_6H_5O_7$ (Fisher Scientific, UK), 2 g CH_3COONa (Univar, USA), 0.1 g $MgSO_4.7H_2O$ (Fisher Scientific, UK), 0.05 g $MnSO_4.H_2O$ (Univar, USA), 2 g K_2HPO_4 (Univar, USA), 50 g reducing sugar from tapioca

starch hydrolysate, and distilled water to make 1 L medium. Final pH was adjusted to 6.6 ± 0.2 by 1 M NaOH. Lactic acid fermentation was allowed at 37°C for 72 h under static condition. Lactic acid, remaining sugar, and dry weight were then measured by the methods described in the sample analysis section.

Sample analysis

After fermentation ceased, the pH level of fermentation broth was measured using a pH meter (Metrohm Siam, Thailand). Then, the pH level was adjusted to 2.0 by 4 M HCl to release free lactic acid from calcium lactate salt. Subsequently, distilled water was added to make equal volume of each sample. For dry biomass analysis, samples were centrifuged at 12,000xg for 5 min and washed twice with distilled water. The sediments were then allowed to dry at 80°C for 24 h and put in a dessicator for 1 h before weighing. Lactic acid concentration in the supernatant was analyzed by the method of Barker and Summerson (1941) while reducing sugar concentration was estimated by DNS method (Miller, 1959). The lactic acid yield per sugar consumption ($Y_{L/S}$) and yield of lactic acid production per biomass formation ($Y_{L/X}$) were calculated.

Effect of $CaCO_3$ on lactic acid production

An optimal concentration of $CaCO_3$ on lactic acid production was evaluated by varying $CaCO_3$ concentration at 0, 50, 75, and 100 g/l in fermentation medium with 50 g/l reducing sugar supplied by tapioca starch hydrolysate. The experiment was carried out triplicately at 37°C for 72 h under static condition.

Effect of yeast extract and $MnSO_4.H_2O$ on lactic acid production

The general full factorial design was created with two independent variables, yeast extract and $MnSO_4.H_2O$, to determine their effects on lactic acid production. Response surface methodology (RSM) was used to analyze the data. The variables involved in this study were described below.

Variables used in the optimization of lactic acid production by RSM

(a) Fixed variables
- Calcium carbonate (50 g/l)
- Reducing sugar (50 g/l)
- Temperature (37°C)
- Time (72 h)

(b) Dimensional (uncoded) independent variables

Variables	Designate	Units	Variation levels
Yeast extract concentration	YE	g/l	(0, 5, 10, 15, 20)
$MnSO_4.H_2O$ concentration	$MnSO_4.H_2O$	g/l	(0.00, 0.05, 0.10)

(c) Dimensionless (coded) independent variables

Variables	Designate	Units	Variation levels
Yeast extract concentration	x_1	dimensionless	(-2, -1, 0, 1, 2)
$MnSO_4.H_2O$ concentration	x_2	dimensionless	(-1, 0, 1)

(d) Dependent variables

Variables	Designate	Units
Lactic acid concentration	Y	g/l
Lactic acid yield	$Y_{L/S}$	g/g

The coded independent variables X_1 and X_2 were defined, for statistical purposes, as follow:

$$x_1 = \frac{YE-10}{5} \quad (1)$$

$$x_2 = \frac{MnSO_4H_20\text{-}0.05}{0.05} \quad (2)$$

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

$$Y = b_0 + \Sigma\, b_i x_i + \Sigma\, b_{ii} x_i^2 + \Sigma\, b_{ij} x_i x_j \quad (3)$$

Where Y is the predicted response of dependent variable, lactic acid concentration and lactic acid yield; 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 = 1-2); X_i and X_j are the independent variables (i, j = 1-2). According to the full factorial design with five levels of yeast extract and three levels of $MnSO_4.H_2O$, fifteen treatments of lactic acid fermentation were conducted in triplicate.

Results and Discussion

Effect of $CaCO_3$ on lactic acid production

Effect of $CaCO_3$ on pH level, lactic acid production, $Y_{L/S}$ and $Y_{L/X}$ by *L. casei* TISTR 453 after 72 h fermentation at 37°C was illustrated in Table 1. An initial concentration of reducing sugar, obtained from tapioca starch hydrolysate, was set at 50 g/l. The result suggested that $CaCO_3$ addition significantly improved the starter culture efficiency on lactic acid production since all treatments with $CaCO_3$ addition provided higher lactic acid concentration comparing to the treatment without $CaCO_3$ ($P<0.05$). The result was in an agreement with the works of Kotzamanidis et al. (2002), and Panda and Ray (2008) who reported that $CaCO_3$ efficiently increased lactic acid production when appropriate amounts were used. Our current research revealed that at 50, 75, and 100 g/l $CaCO_3$, lactic acid concentrations were obtained at 31.15, 33.38, and 34.15 g/l, respectively. Those were approximately twice the amount of lactic acid achieved when $CaCO_3$ was omitted. Similarly, higher $Y_{L/S}$ and $Y_{L/X}$ were observed when $CaCO_3$ was added. In addition, a slower reduction of fermentation pH level was associated with $CaCO_3$ plus medium, providing a pH range between 4.97 and 6.5 for the entire fermentation period (an initial pH level of each fermentation medium was set around 6.6 by $CaCO_3$ and/or 1 M NaOH solution). This is beneficial to the starter culture since a good pH condition was generated. In general, the optimum pH levels for lactic acid production lie between 5.0 and 7.0 (Hofvendahl and Hahn-Hägerdal, 2000). On the other hand, the medium pH dropped dramatically in the treatment without $CaCO_3$ supplementation and the final pH was observed at 3.73 which was out of optimal pH range for lactic acid production by most lactic acid bacteria.

Table 1. Effect of $CaCO_3$ on pH, lactic acid production, $Y_{L/S}$, and $Y_{L/X}$ after fermentation by *L. casei* TISTR 453 with 50 g/l initial concentration of reducing sugar supplied by tapioca starch hydrolysate

$CaCO_3$ (g/l)	pH	Lactic acid (g/l)	$Y_{L/S}$ (g/g)	$Y_{L/X}$ (g/g)
0	$3.73^{a} \pm 0.01$	$15.98^{a} \pm 0.16$	$0.53^{a} \pm 0.16$	$1.82^{a} \pm 0.14$
50	$4.97^{b} \pm 0.01$	$31.15^{b} \pm 0.20$	$0.72^{b} \pm 0.25$	$3.12^{b} \pm 0.27$
75	$5.27^{c} \pm 0.01$	$33.38^{b} \pm 0.34$	$0.69^{b} \pm 0.07$	$3.30^{c} \pm 0.21$
100	$5.46^{d} \pm 0.02$	$34.15^{b} \pm 0.23$	$0.71^{b} \pm 0.21$	$3.09^{b} \pm 0.61$

$^{a.b.c,d}$Values with different letters in the same column are significantly different ($P < 0.05$). The data are expressed as mean ± standard deviation.

Among the three levels of $CaCO_3$ concentration, however, the difference between lactic acid and $Y_{L/S}$ are not statistically significant (P>0.05). Besides, too high concentration of $CaCO_3$ caused quite a few disadvantages, i.e. the inconvenience on dry weight measurement and the environmental adverse impact caused by extra amounts of disposal. The solubility of $CaCO_3$ is very poor. Thus, it may interfere with biomass measurement if its residues remain. The $Y_{L/x}$ of 50 g/l $CaCO_3$ was high as well. Accordingly, 50 g/l of $CaCO_3$ was chosen to study the effect of yeast extract and $MnSO_4.H_2O$ on lactic acid production by RSM in the following experiment.

Effect of yeast extract and $MnSO_4.H_2O$ on lactic acid production evaluated by RSM

Lactic acid concentration and $Y_{L/S}$ were analyzed and the results were shown in Table 2. Then, the multiple regression analysis was applied to experimental data of lactic acid concentration yielding the regression coefficients, t-value, and probability value (P-value) revealed in Table 3.

Table 2. Full factorial design for the optimization of yeast extract and $MnSO_4.H_2O$ on lactic acid production and $Y_{L/S}$ by *L. casei* TISTR 453 and experimental values of the two responses

Run	Yeast extract* (x_1)	$MnSO_4.H_2O$* (x_2)	Lactic acid (g/l)	$Y_{L/S}$ (g/g)
1	-2	-1	11.24	0.31
2	-2	0	14.50	0.39
3	-2	1	16.58	0.43
4	-1	-1	17.11	0.45
5	-1	0	33.78	0.77
6	-1	1	32.30	0.72
7	0	-1	22.94	0.58
8	0	0	37.10	0.77
9	0	1	33.27	0.73
10	1	-1	29.84	0.67
11	1	0	39.09	0.79
12	1	1	37.12	0.77
13	2	-1	37.05	0.77
14	2	0	41.94	0.85
15	2	1	39.56	0.79

*Values of yeast extract and $MnSO_4.H_2O$ presented were coded values.

The output interpretation was made following the procedure of Box and Draper (2007), Myers and Montgomery (2002). The P-value indicated the significance of each variable coefficient. The smaller the P-value, the more significant the corresponding coefficient is. According to the multiple regression analysis output in Table 3, the probability of the linear and squared terms of yeast extract and $MnSO_4.H_2O$ are significant ($P<0.05$), interpreting that yeast extract and $MnSO_4.H_2O$ affected lactic acid production. Therefore, the coefficients of those variables were included in the quadratic equation whereas the coefficient of the interaction term ($x_1 x_2$) was neglected since its P-value was larger than 0.05, implying that the interaction effect of yeast extract and $MnSO_4.H_2O$ was not significant. Hence, the following quadratic equation was yielded.

Table 3. Regression coefficients, t-value, and P-value for lactic acid production using general full factorial design and analyzed by RSM

Variable	Designate	Coefficients	Std. error coefficient	t-value	P-value
Constant	-	35.8591	1.9039	18.834	0.000
YE	x_1	5.8440	0.6397	9.135	0.000
Mn	x_2	4.0650	1.1081	3.669	0.005
YE x YE	x_1^2	-1.2886	0.5407	-2.383	0.041
Mn x Mn	x_2^2	-5.5810	1.9192	-2.908	0.017
YE x Mn	$x_1 x_2$	-0.6785	0.7835	-0.866	0.409

$R^2 = 0.925$, YE = yeast extract, Mn = $MnSO_4.H_2O$

$$Y=35.859+5.844X_1+4.065X_2-1.289X_1^2-5.581X_2^2 \quad (4)$$

Where Y is the predicted lactic acid concentration, X_1 and X_2 are the coded values of yeast extract and $MnSO_4.H_2O$ concentration, respectively.

Effects of the linear terms, squared terms, and interaction terms were summarized in the analysis of variance for lactic acid production in Table 4. The small P-value of the squared term (p=0.014) suggested the curvature in the response surface. The insignificance of the interaction effect was confirmed by the high P-value (P = 0.409).

Table 4. Analysis of variance for lactic acid production

Source	df	Seq SS	Adj SS	Adj MS	F-value	P-value
Regression	5	1372.58	1372.58	274.52	22.36	0.000
Linear	2	1189.81	1189.81	594.91	48.45	0.000
Square	2	173.56	173.56	86.78	7.07	0.014
Interaction	1	9.21	9.21	9.21	0.75	0.409
Residual Error	9	110.50	110.50	12.28		
Total	14	1483.08				

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

From the model generated (Equation 4), lactic acid concentrations produced by *L. casei* TISTR 453 at different combination levels of yeast extract and $MnSO_4.H_2O$ were predicted and the three dimensional surface plot is shown in Figure 1. It was evident that yeast extract satisfactorily enhanced lactic acid production while only certain levels of $MnSO_4.H_2O$ promoted the efficiency of *L. casei* TISTR 453 on the bioconversion of sugar into lactic acid. The result was in an agreement with the work of Fitzpatrick et al. (2001). Too high concentrations of $MnSO_4.H_2O$ adversely affected lactic acid fermentation. In Figure 1, the amounts of $MnSO_4.H_2O$ which positively influenced lactic acid production were not higher than 0.05 g/l. Over this point, the product formation declined.

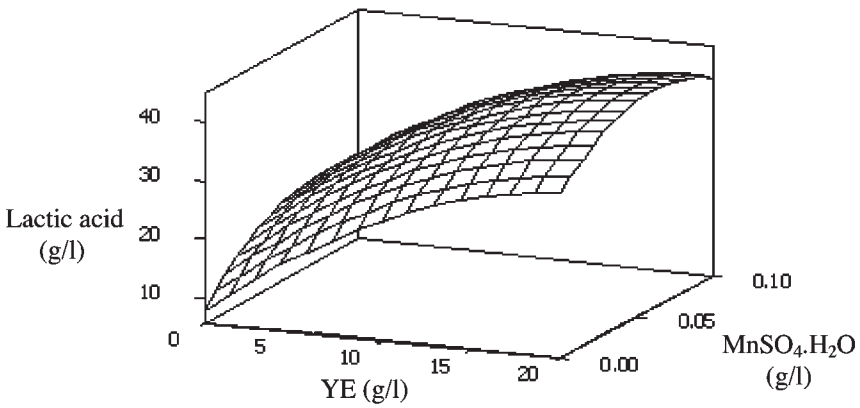

Figure 1. Surface plot of predicted lactic acid production by *L. casei* TISTR 453 when the levels of YE and $MnSO_4.H_2O$ were varied in the range of 0-20 g/l and 0.00-0.10 g/l, respectively

Within the ranges of yeast extract and $MnSO_4.H_2O$ levels studied, optimum levels of yeast extract and $MnSO_4.H_2O$ were at 20 and 0.05 g/l, respectively, which yielded the maximum predicted lactic acid concentration at 42.39 g/l. Figure 2 presents a correlation between experimental and predicted values of lactic acid formation. All points were located around the diagonal line (R^2 = 0.925), indicating that the model efficiently predicted the production of lactic acid by *L. casei* TISTR 453.

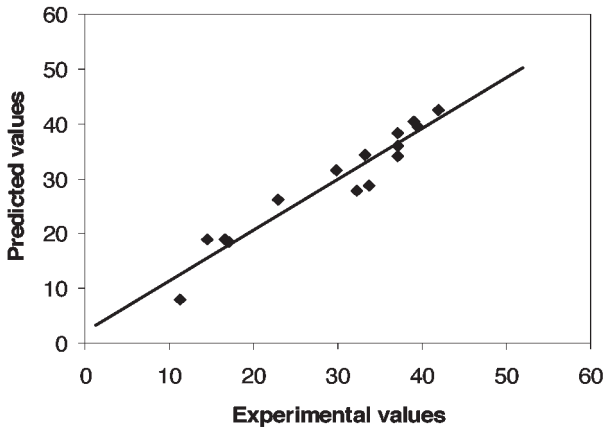

Figure 2. Experimental vs. predicted values plot for lactic acid production by *L. casei* TISTR 453 when levels of yeast extract and $MnSO_4.H_2O$ were varied in the range of 0-20 g/l and 0.00-0.10 g/l, respectively. The R^2 value was 0.925.

$Y_{L/S}$ was analyzed the same way as that described for lactic acid concentration and the maximum $Y_{L/S}$ predicted by the model was 0.84 g/g substrate utilized when yeast extract and $MnSO_4.H_2O$ were supplied at 20 and 0.05 g/l, respectively (data not shown). The predicted value was very close to the experimental data shown in Table 2 at the same concentration of yeast extract and $MnSO_4.H_2O$ (0.85 g/g substrate utilized).

Conclusion

Tapioca starch hydrolysate was a good substrate for lactic acid production by *L. casei* TISTR 453. $CaCO_3$, yeast extract, and $MnSO_4$ were needed in fermentation medium. The optimized medium resulted in significantly higher lactic acid concentration and $Y_{L/S}$ than the initial (un-optimized) fermentation medium.

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