รายงานโครงงานวิจัย

เงินงบประมาณประจำปี 2555

เรื่อง

การใช้ยีสต์ออโตไลเสทเป็นแหล่งไนโตรเจนทางเลือกสำหรับการผลิตกรดโพรพิโอนิกโดยเชื้อ *Propionibacterium acidipropionici* ATCC 422 ที่ถูกตรึงด้วยสารสกัดเพคตินหยาบจากใบกรุงเขมา (*Cissampelos pareira* L.)

Using Yeast Autolysate as an Alternative Nitrogen Source for Propionic Acid Production by Immobilized *Propionibacterium acidipropionici* ATCC 422 with Pectin Crude Extracts from Krung Kha Mao Leaves (*Cissampelos pareira* L.)

โดย

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สารบัญ

เรื่อง **หน้า**

บทคัดย่อภาษาไทย

งานวิจัยนี้เลือกใช้กากน้ำตาลเป็นแหล่งคาร์บอนราคาถูกและยีสต์ออโตไลเสทเพื่อเป็นแหล่งไนโตรเจนทางเลือกสำหรับศึกษาการผลิตกรดโพรพิโอนิกโดย *Propionibacterium acidipropionici* TISTR 422 และได้ใช้ Plackett-Burman Design ในการศึกษาสภาวะที่สามารถผลิตกรดได้สูงสุด การทดลองได้กระทำใน Erlenmeyer flasks ขนาด 125 มิลลิลิตร และมีปริมาณอาหาร 63 มิลลิลิตร ในอาหารเติมแคลเซียมคาร์บอเนตร้อยละ 1 (น้ำหนักต่อปริมาตร) เพื่อรักษาระดับของพีเอชในอาหาร หลังจากผ่านการหมัก เป็นเวลา 7 วัน ปริมาณกรดสูงสุดที่ได้ 30.84 กรัมต่อลิตร และผลผลิตของกรดโพรพิโอนิกได้ 4.4 กรัมต่อลิตรต่อวัน ซึ่งสภาวะที่เหมาะสม คือ ปริมาณน้ำตาลในกากน้ำตาล 20 กรัมต่อลิตร ปริมาณยีสต์สกัด 10 กรัมต่อลิตร และปริมาณยีสต์ออโตไลเสท 10 กรัมต่อลิตร

คำสำคัญ Plackett-Burman design กรดโพรพิโอนิก *Propionibacterium acidipropionici* TISTR 422 กากน้ำตาล ยีสต์ออโตไลเสท

ABSTRACT

The production of propionic acid by *Propionibacterium acidipropionici* TISTR 422 was investigated by using molasses as a cheap carbon source and yeast autolysate as an alternative nitrogen source. A Plackett-Burman design was used to determine maximum propionic acid production. The assay was performed in 125 ml Erlenmeyer flasks containing 63 ml of production medium, 1% (w/v) calcium carbonate was added to the production medium in order to maintain the pH constant. The maximum propionic acid concentration reached to 30.84 $g.l^{-1}$ and propionic productivity was 4.4 $g.l^{-1}d$ after 7 days, which corresponded to 20 $g.l^{-1}$ total sugar from molasses, 10 $g.l^{-1}$ of yeast extract and 10 $g.l^{-1}$ of yeast autolysate.

Keywords: Plackett-Burman design, Propionic acid, *Propionibacterium acidipropionici* TISTR 422, Molasses, Yeast autolysate

INTRODUCTION

Propionic acid is an important chemical that is widely used as a raw material in different industries (11, 19). Currently, almost propionic acids produce by petrochemical process, but propionic acid biosynthesis is expected to be a promising option due to its renewable raw sources and the overall increasing consumer demand. Although, there has been an interest to produce propionic acid from biomass by fermentation with propionibacteria, but the relatively low propionic acid concentration, yield and production rate from the fermentation have been the major barriers for economical applications (54).

A number of by-products and raw materials from the food and/or agriculture industries have been employed for microorganism growth due to their considerable availability and low cost (13). Several carbon sources have been patented for producing propionic acid by fermentation such as glucose (20, 21, 25, 34, 54), lactose (29), xylose (10), sucrose (45), and glycerol (6, 14, 16, 25, 31, 37, 58, 61). The cheap raw materials, such as whey (9, 11, 29,31), hemicelluloses (38, 48) hydrolyzed corn meal (27). All these were also applied for the propionic acid production. Furthermore molasses, the by-product of the sugar industry, contained about 50% (w/w) total sugar (sucrose, glucose and fructose) were also utilized for the propionic acid production as a cheap carbon source (19).

Yeast autolysate and yeast extract are the main nitrogen source used for the production of propionic acid, which is rich in free amino acid, proteins, vitamins, and fiber. Therefore, it is good for use as supplements in culture media (8). Furthermore, molasses and yeast autolysate are prominent composition for culture media in fermentative processes due to the high content of sugar and nitrogen respectively (7).

Response surface methodology (RSM) is the collection of statistical techniques for experiment design, model development, evaluation factor, and optimum condition search. Now it is extensively applied in the optimization of medium composition, conditions of enzymatic hydrolysis, fermentation, and food manufacturing processes. RSM design was used for further study of the influences of major factor and interaction between them on the response value, which is based on the result of sole-experiment and Plackett-Burman (PB) design. PB design is a method of choice for initial screening of medium components (51).

However, no research used statistically based experimental design for the screening of the media component to improve the propionic acid production from *P.acidipropionici* TISTR 422. The aim of this study was to investigate the use of molasses as a carbon sources in culture medium to obtain the optimum conditions for biomass production by *Candida utilis* TISTR 5046. In this study, we investigate the

suitable condition for propionic acid production by *P. acidipropionici* TISTR 422 using molasses and yeast autolysate as carbon and nitrogen sources in Plackett-Burman design method.

MATERIALS AND METHODS

Microorganisms

C. utilis TISTR 5046 obtains from microbiology laboratory of KMITL, was used in biomass production because of its ability to utilize a variety of carbon source and to support high protein (2, 18, 41, 46, 47). The culture was stored in YM medium at 4 °C and reactivated every 2 mounths (49, 50, 55, 60).

P. acidipropionici TISTR 422 obtains from Thailand Institute of Scientific and Technological Research (TISTR). This strain to produce propionic acid, under anaerobic condition (54, 58, 61). The culture was stored in MRS medium at 4 °C (11, 12, 22) and reactivated every 2 mounths.

Medium and growth conditions

Preculture medium was used as inoculum medium for *C. utilis* TISTR 5046 consisted of peptone 5 $g.l^{-1}$, yeast extract 3 $g.l^{-1}$, malt extract 3 $g.l^{-1}$ and glucose 3 $g.l^{-1}$ (55, 60). The inoculum was grown at 30 °C with pH 6.0 for 24 hours on a shaker at 150 rpm (15, 46, 47, 50, 60).

The fermentation medium for *C. utilis* TISTR 5046 consisted of the hydrolyzed molasses containing 1% (w/v), KH_2PO_4 5 $g.l^{-1}$, $(NH_4)_2SO_4$ 5 $g.l^{-1}$, $CaCl_2$ 0.13 $g.l^{-1}$, $MgSO_4.7H_2O$ 0.5 $g.l^{-1}$, and yeast extract 0.5 $g.l^{-1}$ (2, 46) at 30 °C, pH of the medium was adjusted to 4.5-5.5 and shaking at 150 rpm until the 48 h. The sample were measured total sugar content by the Dubois's process (17, 33 41), free amino acid by HPLC (15), total nitrogen and protein content by Kjeldaht method (41, 60).

The inoculum of *P. acidipropionici* TISTR 422, was prepared by transfer a loopfull to 50 ml of growth MRS medium in Erlenmeyer flasks 250 ml (11). The MRS growth medium was made up of peptone 10 $g.l^{-1}$, beef extract 10 $g.l^{-1}$, yeast extract 5 $g.l^{-1}$, glucose 20 $g.l^{-1}$, tween80 1 ml, K_2HPO_4 2 $g.l^{-1}$, sodium acetate 5 $g.l^{-1}$, tri-ammonium citrate 2 $g.l^{-1}$, $MgSO_4.7H_2O$ 0.2 $g.l^{-1}$and $MnSO_4.4H_2O$ 0.2 $g.l^{-1}$ (11, 12, 22, 43), pH of the medium was adjusted to 6.5 (23, 37, 54), the inoculated medium was inocubated at 30 °C for 48 hour (14, 23, 37, 38, 62) at static state. A total of 5% (v/v) (11,11,8) of the inoculum was transferred to 125 ml Erlenmeyer flasks containing 63 ml of production medium,1% (w/v) calcium carbonate was added to the production medium in order to maintain the pH constant (13). Sample were taken from culture 0 hour until the 360 hour were measured pH value, total sugar content by the Dubois's process and analyse for propionic acid and acetic acid by high performance liquid chromatography (HPLC) (11).

Molasses hydrolysis

Molasses were adjusted to pH 3.0 (32, 52, 57) by addition of 20% H_2SO_4 (w/v). Then the molasses was remained at 80 °C with water batch for 20 min (12), all of the sucroses were hydrolyzed for the glucose and fructose. The hydrolyzed molasses were centrifuged at 10,000×g for 10 min. The supernatants were collected and adjusted to pH 6.5 with 10 M NaOH (18, 32, 52) and supernatants sterilized at 121 °C for 15 min.

Analysis

The cell dry weight was determined by the following way: yeast cell were harvested by centrifugation, washed twice with distilled water and dryed at 105 °C overnight until constant weight was reached (15, 33, 50, 51), the total sugar content of the medium was determined by Dubois's process (17, 33, 51), free amino acid by HPLC (15), total nitrogen and protein content were determined with the Kjeldahl method (5, 41, 47, 60)

Cell growth was estimated by measuring the optical density of cell suspensions at 600 nm in a spectrophotometer (DR/4000, HACH Co., Ltd) (37, 54, 58, 61). The supernatants of the samples which had been centrifuged at 10,000×g for 10 min were analyzed by HPLC. Propionic acid and acetic acid were quantified by filtering the samples through 0.45 μm cellulose membranes on to an inerstsil C8-3 column (1.6×250 nm) and operated at room temperature, using 5mM H_2SO_4 (14, 23, 61) as the mobile phase. The wave length of the UV detector was 210 nm and the flow rate was 0.1 $ml.min^{-1}$ (11). Total sugar was determined by the Dubois's process (17) and measured pH value by pH meter.

Plackett-Burman design

The purpose of the first optimization step was used for screening of the factors that significantly influenced propionic acid production (3, 30). Base on Plackett-Burman design, each variable was examined in two levels: -1 for low level and +1 for high level (59). This design was used to evaluated the important factor that influence the response of twelve assigned factor, were screened including: molasse, temperature, pH, yeast autolysate (nanoproplus), yeast extract, trytic soy broth (TSB), K_2HPO_4, KH_2PO_4, $MnSO_4.4H_2O$, $MgSO_4.7H_2O$, $CaCl_2.6H_2O$ and $CoCl_2.6H_2O$.

RESULTS

The study of *Candida utilis* TISTR 5046 biomass and nitrogen composition analysis for an alternative nitrogen source.

In this study , we interested in the biomass and nitrogen composition in *C. utilis* for using as the candidate of nitrogen source and using for yeast autolysate production . We have been determined the growth rate of yeast cells that related to biomass and yield that is tend to be use as nitrogen source in propionic acid fermentation. At 48 hours, It is suitable for yeast biomass production, but in experiments we have to use the commercial yeast extract and yeast autolysate (nanoproplus^{TM}) as nitrogen source because we have to study the suitable conditions for propionic acid production and after that we will use yeast autolysate from *C. utilis* as nitrogen source instead.

C. utilis was studied for growth using molasses as a sole carbon for an alternative nitrogen source in propionic acid production. Ahmed, S. et al., (2010) reported that molasses, a cheap by-product is widely available from the sugar industry and consist of water, sucrose which is disaccharide most easily utilized by yeast cell, nitrogen source, proteins, vitamins, amino acids, organic acids and heavy metals, and found that 1% molasses gave higher microbial biomass production by sequential culture fermentation of *Arachniotus* sp. and *C. utilis*.

In this study, the pH of the medium during cultivation was maintained at 4.5-5.5, incubated at 30 °C, shaking at 150 rpm. After 48 hours were found suitable for maximum production of biomass (4.29 $g.l^{-1}$), productivity of biomass (2.14 $g.l^{-1}h$) and utilization of sugar (82.44 % w/w) as shown in **Figure 1**. *C. utilis* TISTR 5046 was cultivated in fermentation medium contain concentration molasses 10 $g.l^{-1}$. After 6 hours, the microbial cells started to adapting during lag phase state. Cells were rapidly grown until the 24 hours, then growth were slow and reaches a stationary phase. The total sugar was also determined and the results showed that the consumption of sugar were consistant with the cell dry weight (DCW). **Figure 1**, shows that highest amount of sugar consumption is 10 $g.l^{-1}$ at 4.5-5.5 and 30 °C. The experiment were initially investigation for sugar consumption at a constant retention time of 24 hours.

The part of autolysate experiment was done at pH 4.5-5.5, 30 °C for 48 hours. Yeast suspension was centrifuged at 10,000×g for 10 min to remove the supernatant, washed twice with distilled water and then, adjusted pH of yeast suspension at 5.5-6.0, incubated in water bath at 50 °C for 24 hours reaction time of autolysis, autolysed yeast extract of *C. utilis* TISTR 5046 was concentrated by rotary vacuum evaporator and analysed total nitrogen and protein content of samples as shown in **Table 1** and the analysis amino acid was showed in **Table 2**.

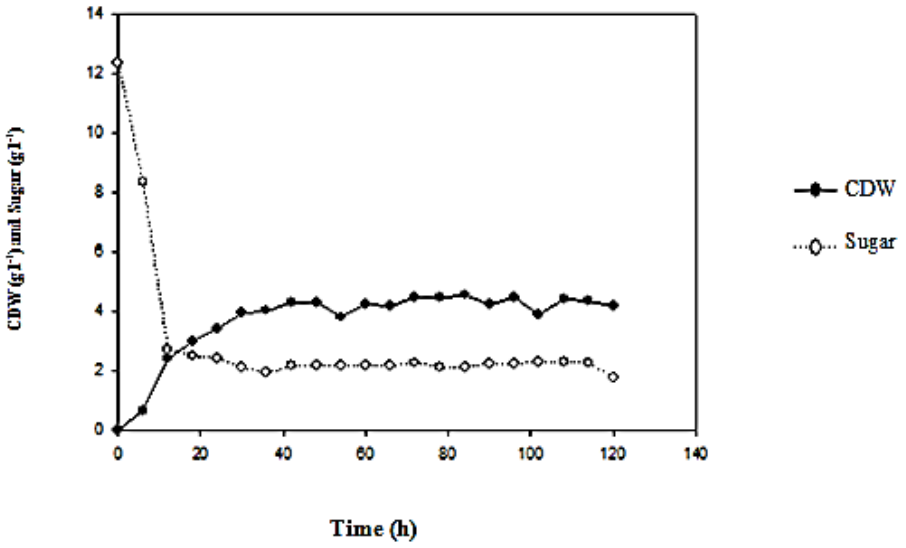

Figure 1. Growth curves of *C. utilis*TISTR 5046 in molasses

Table 1. Chemical compositions of yeast extract from *C. utilis* TISTR 5046, nanoproplus™ and commercials yeast extract

Chemical compositions	**Yeast extract form *C. utilis* TISTR 5046**	**Nanoproplus™**	***Commercials yeast extract**
Total nitrogen (%)	2.12	8.41	13.66
Protein content (%)	13.25	52.56	84.38

Nanoproplus™ from Specialty Biotech Co;Ltd.

*Commercials yeast extract from Himedia Laboratories Pvt.Ltd

Table 2. Amino acid compositions of yeast extract from *C. utilis* TISTR 5046, nanoproplus™ and commercials yeast extract

Amino acids	Compositions (mg.100 ml^{-1})		
	Yeast extract from *C.utilis* TISTR 5046	Nanoproplus™	*Commercials yeast extract
Aspartic acid	0.82	0.24	26.75
Threonine	0.06	0.56	25.83
Serine	0.09	0.10	26.48
Glutamic acid	0.87	8.42	85.85
Proline	0.14	2.44	13.62
Glycine	0.08	1.71	17.39
Alanine	0.13	8.49	53.18
Cystine	0.24	0.62	-
Valine	0.04	2.91	34.27
Methionine	0.20	0.54	7.86
Isoleucine	0.03	1.45	27.73
Leucine	0.03	2.56	47.63
Tyrosine	1.02	3.38	16.20
Phenylalanine	1.08	30.81	27.91
Histidine	0.15	0.51	3.41
Lysine	0.36	1.32	25.67
Arginine	0.11	0.29	20.94
Tryptophan	0.12	0.12	4.86

Nanoproplus™ from Specialty Biotech Co;Ltd.

*Commercials from Himedia Laboratories Pvt.Ltd

Screening of significant variables by Plackett-Burman design

Plackett-Burman design was used in this study for selecting the significant variables for propionic acid production. The concentration of molasses was 20 $g.l^{-1}$ (w/v), yeast autolysate 10 $g.l^{-1}$ and yeast extract 10 $g.l^{-1}$. Propionic acid production reached the maximal value of 30.84 $g.l^{-1}$ within 168 hours of fermentation, with the productivity of 4.4 $g.l^{-1}h$ (**Table 3**). While, molasses concentration 40 $g.l^{-1}$ (w/v),yeast autolysate 5 $g.l^{-1}$ and yeast extract 5 $g.l^{-1}$, gave propionic acid production of 36.42 $g.l^{-1}$ in 264 hours with the productivity of 3.3 $g.l^{-1}$ h, which is a lower production rate (data not shown). Moreover, when nitrogen concentration increase, it led to a reduction in fermentation time, and reached maximal value propionic acid production. **Table 3** displayed the Plackett-Burman design (coded values) of the 20 experiment with 12 variables (F_1 = molasses, F_2 = temperature, F_3 = pH, F_4= yeast autolysate (nanoproplus), F_5 = yeast extract, F_6 = TSB, F_7 = K_2HPO_4, F_8 = KH_2PO_4, F_9 = $MnSO_4.4H_2O$, F_{10} = $MgSO_4.7H_2O$, F_{11} = $CaCl_2.6H_2O$, F_{12} = $CoCl.6H_2O$).

ANOVA of the model is given in **Table 4**. The model F-value of 3.69 implies that the model is significant. The goodness of fit of the model was checked by determination coefficient (R^2). In this study, the R^2 value was calculated to be 0.8312. A regression model with R^2 colsed to 1.0 is considered as having a very high correlation, whereas Adjusted R^2 of 0.6061 implies that confirmed the significance of the mode as well.

Table 3. Plackett-Burman design (coded values) with the respective results

Run	Independent variables												Propionic acid (g. l^{-1})
	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}	F_{11}	F_{12}	
1	+1	-1	-1	-1	-1	+1	+1	-1	+1	+1	-1	-1	23.28
2	+1	-1	-1	+1	+1	+1	+1	-1	+1	-1	+1	-1	25.43
3	+1	-1	+1	-1	-1	-1	-1	+1	+1	-1	+1	+1	24.74
4	+1	+1	-1	+1	+1	-1	-1	+1	+1	+1	+1	-1	20.90
5	-1	-1	+1	+1	+1	+1	-1	+1	-1	+1	-1	-1	17.66
6	+1	+1	-1	-1	+1	+1	+1	+1	-1	+1	-1	+1	17.73
7	-1	-1	-1	+1	+1	-1	+1	+1	-1	-1	+1	+1	14.19
8	+1	+1	+1	+1	-1	+1	-1	+1	-1	-1	-1	-1	19.63
9	+1	-1	+1	+1	-1	-1	+1	+1	+1	+1	-1	+1	23.89
10	-1	+1	+1	-1	+1	+1	-1	-1	+1	+1	+1	+1	26.98
11	+1	+1	-1	+1	-1	+1	-1	-1	-1	-1	-1	-1	16.21
12	-1	-1	+1	+1	-1	+1	+1	-1	-1	+1	+1	+1	17.96
13	-1	+1	+1	-1	-1	+1	+1	+1	+1	-1	+1	-1	20.52
14	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	14.17
15	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	-1	+1	19.07
16	-1	+1	-1	-1	-1	-1	+1	+1	-1	+1	+1	-1	23.27
17	-1	+1	+1	+1	+1	-1	+1	-1	+1	-1	-1	-1	30.84
18	-1	-1	-1	-1	+1	-1	-1	+1	+1	-1	-1	+1	14.63
19	+1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	-1	22.90
20	+1	+1	+1	-1	+1	-1	+1	-1	-1	-1	-1	+1	25.39

Table 4. ANOVA for Plackett-Burman.

Source	Sum of squares	df	Mean square	F-value	p-valuve Prob < F
Model	333.51	12	27.79	3.69	0.0289
F_1-Molasses	21.65	1	21.65	2.88	0.1241
F_2-Temperature	23.52	1	23.52	3.13	0.1109
F_3-pH	86.65	1	86.65	11.51	0.0080
F_4-Yeast autolysate	3.07	1	3.07	0.41	0.5392
F_5-Yeast extract	9.67	1	9.67	1.29	0.2862
F_6-TSB	18.68	1	18.68	2.48	0.1496
F_7-K_2HPO_4	32.79	1	32.79	4.36	0.0665
F_8-KH_2PO_4	31.43	1	31.43	4.18	0.0714
F_9-$MnSO_4.4H_2O$	84.75	1	84.75	11.26	0.0084
F_{10}-$MgSO_4.7H_2O$	3.11	1	3.11	0.41	0.5362
F_{11}-$CaCl_2.6H_2O$	2.32	1	2.32	0.31	0.5924
F_{12}-$CoCl_2.6H_2O$	15.86	1	15.86	2.11	0.1805
Cor Total	402.12	22			

Std. Dev: 2.74; R-Squared: 0.8312; Adj R-Squared: 0.6061; Pred R-Squared: -0.3750; PRESS: 552.93; Adeq: 6.696

Molasses, temperature, pH, yeast extract, K_2HPO_4, $MnSO_4.4H_2O$, $MgSO_4.7H_2O$, and $CaCl_2.6H_2O$ had positive coefficients while the other three variables showed negative coefficients. Molasses was used as the dominant nutrients in production of propionic acid. Yeast extract as the sole nitrogen source in medium, contains abundant of amino acid, minerals and vitamin, which are necessary for cell growth and increased propionic acid production. Altaf et al. (2006) reported that peptone and yeast extract are the main nitrogen sources used for the production of lactic acid and if alternative source were used, the final product would be smaller and the fermentation time would increase. The effect of yeast extract was kept constant at low level because of the supplement with yeast autolysate (nanoproplus^{TM}) in the medium. On the other hand, high concentration of nitrogen may lead to cell death and inhibition of the product.

K_2HPO_4 is phosphate source in fermentation medium plays a key role in enhancing of microorganism growth, Honorato et al. (2007) revealed that the use of K_2HPO_4 is reported to provide K^+ and phosphate (PO_4^-) for microorganism growth and also acts as a buffering agent in the medium.

The coefficient of $MnSO_4 4H_2O$, $MgSO_4.7H_2O$, and $CaCl_2.6H_2O$ were positive, suggested that it remain constant at high level, which mean that this chemical reagent is trace amount in culture medium but necessary and important for cell growth. Yeast autolysate (nanoproplus^{TM}) has negative coefficient, thus in the next study had to reduce or keep in contant level the concentration of mineral ion. Whereas, tryticase soy broth (TSB) showed a negative coeffient, in comparison with that of yeast extract and yeast autolysate (nanoproplus^{TM}), it contribution was the least significant in production of propionic acid and therefore TSB can be excluded from further experiment. Furthermore, KH_2PO_4 and $CoCl.6H_2O$ were showed negative coefficients, therefore their lower level may be suggested for further experiments shown in **Table 5**.

Table 5. Coefficient of each variable, confidence interval (CI) at 95% confidence level based on 't' statistic and sum of the squares as percentage (SS%) for production of propionic acid in 12 variable Plackett-Burman design.

Factor	Coefficient	95% CI Low	95% CI High
F_1-Molasses	1.04	-0.35	2.43
F_2-Temperature	1.08	-0.30	2.47
F_3-pH	2.08	0.69	3.47
F_4-Yeast	-0.39	-1.78	1.00
F_5-Yeast extract	0.70	-0.69	2.08
F_6-TSB	-0.97	-2.35	0.42
F_7-K_2HPO_4	1.28	-0.11	2.67
F_8-KH_2PO_4	-1.25	-2.64	0.13
F_9-$MnSO_4.4H_2O$	2.06	0.67	3.45
F_{10}-$MgSO_4.7H_2O$	0.39	-0.99	1.78
F_{11}-$CaCl_2.6H_2O$	0.34	-1.05	1.73
F_{12}-$CoCl_2.6H_2O$	-0.89	-2.28	0.50

DISCUSSION

According to the study of *C. utilis* TISTR 5046 growth rate, at 48 hours showed the highest growth rate and it is the suitable period for harvest yeast cells. At this state, yeast cell biomass reached 4.29 $g.l^{-1}$ which is the highest value and the percentage of sugar utilization is 82.44 %. Zhao, G. et al (2010) studied production of single cell protein using waste capsicum powder produced during capsanthin extraction, *C. utilis* 1769 was chosen as the biomass producer because of its highest SCP formation 6.8 $g.l^{-1}$.

C. utilis TISTR 5046 was chosen as the biomass producer because of its highest biomass formation. Nigam (2000) reported *C. utilis* has been frequently used in SCP production because of its ability to utilize a variety of carbon sources and to support high protein yield and used in yeast autolysate production by *C. utilis* were performed at 50 ^{o}C for 24 hours. After that yeast autolysate were harvested and concentrated for chemical composition analysis as shown in **Table 1** and **Table 2**. Ahmed, S. et al. (2010) revealed that the production of microbial biomass protein by sequential culture fermentation of *Arachiotus* sp. and *C. utilis* found that the mixed microbial biomass protein in fermentation contained 16.41% of true protein, 23.51% of crude protein, 19.9% of crude fiber, 12.11% of ash and 0.12% of RNA content, while the amino acid profile of final mixed microbial biomass protein showed that it was enriched with essential amino acids (aspartic acid, threonine, serine, gluatamic acid, proline, glycine, alanine, valine, methoinine, isoleucine, leucine, tyrosine, phynylalanine, lysine, histidine and arginine). Dimova, N.D. et al. (2010) studied production of candida biomass from hydrolysed agricultural biowaste, revealed that *C. tropicalis* and *C. utilis* grown on agricultural as the substrate, are promising yeast strains for the production of single cell protein and biomasses from *C.tropicalis* and *C. utilis* may be used as a source of protein after sulfur amino acid enrichment.

However, the study of yeast growth rate were useful in the experiment of yeast autolysate production in the next study, it is rich in free amino acid, protein and other, which can be a good source of supplement. From the second experiments, yeast autolysate is possible to be the alternative nitrogen sources because of their essential amino acids and suitable for use in propionic acid production, consistant with the research work.

Wood and Holzapfel (1995) found that the nitrogen source is a major factor of influence on the growth of Lactobacillus. Cristian J. et al. (2009) studied the production of D(-) lactic acid from *Lactobacillus* LMI8 sp. by using 2 low cost nitrogen sources: corn steep liquor (CSL) and yeast autolysate (YA). Maximal production of lactic acid was 41.42 $g.l^{-1}$ and a value located at the central point, which corresponded to 15 $g.l^{-1}$ of CSL and 5 $g.l^{-1}$ of YA. Selmer-Olsen and Sorhaug (1998) reported that yeast extract is an excellent source of B complex vitamin and often used to provide these factor to the bacteriological culture media, which are often considered indispensable to obtaining faster growth and production rate of lactic acid by lactic bacteria. Moreover, the increase in the nitrogen concentration in the fermentative medium led to a reduction in fermentation time. De Lima et al. (2009) reported about high concentration of nitrogen, Which can lead to cell death. The previous experiments, the result consistence with the report of the using alternative nitrogen for propionic acid production from others study.

Under optimized conditions of this studied, the best result for propionic acid production (30.84 $g.l^{-1}$) was obtained after 168 hours with 20 $g.l^{-1}$ of molasses, 10 $g.l^{-1}$ of yeast autolysate (nanoproplus^{TM}), 10 $g.l^{-1}$ yeast extract. Thus, the use of molasses for fermentation by *P.acidipropionici* TISTR 422 is feasible and yield considerable propionic acid production, requiring supplementation with a cheap nitrogen source (yeast autolysate)

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REFERANCES

(1) Adoki, A. 2008. Factor affecting yeast growth and protein yield production from orange, plantain and banana wastes processing residues using *Candida* sp. Journal of Biotechnology. 7(3): 290-295.

(2) Ahmed, S., Ahmad, F. and Hashmi, A.S. 2010. Production of microbial biomass protein by sequential culture fermentation of *Arachiotus* sp. and *Candida utilis*. Journal of Bot. 42(2): 1225-1234.

(3) Ali, H.K.Q. and Zulkuli, M.M.D. 2011. Application of Plackett-Burman design for screening the media component for citric acid production from paddy straw using solid-state fermentation. Journal of Chemtech Research Coden (USA): IJCRGG. 3(2): 1015-1019

(4)Altaf, Md., Naveena, B.J., Venkateshwar, M., Vijay Kumar, E. and Reddy, G. 2006. Single step fermentation of starch to L(+) lactic acid by *Lactobacillus amylophilus* GV6 in SSF using inexpensive nitrogen sources to replace peptone and yeast extract optimization by RSM. Journal of Process Biochem. 41: 465-472.

(5) AOAC. 2005. Official methods of analysis, Association of official analytical chemists. Washington, DC, USA. 18th edition.

(6) Barbirato, F., Chedaille, D. and Bories, A. 1997. Propionic acid fermentation from glycerol: comparison with conventional substrates. Journal of Appl. Microbiol. Biotechnol. 47: 441-446.

(7) Beaulieu, M., Beaulieu, Y., Melinard, J., Pandian, S. and Goulet, J. 1995. Influence of ammonium salts and cane molasses on growth of *Alcaligenes entrophux* and production of polyhydroxybutyrate. Journal of Appl. Environ. Microbiol. 61: 165-169.

(8) Bekatorou, A., Psarianos, C. and Koutinas, A.A. 2006. Production of food grade yeasts. Journal of Food Technol. Biotechnol. 44(3): 407-415.

(9) Blanc, P. and Goma, G. 1989. Propionic acid and biomass production using continuous ultrafiltration fermentation of whey. Journal of Biotech. Lett. 11(3): 189-194.

(10) Carrondo, M.J.T., Crespo, J.P.S.G. and Moura, M.J. 1988. Production of propionic acid using a xylose utilizing *Propionibacterium*. Journal of Biochem. Biotechnol. 17: 295-312.

(11) Choojun, S. and Yoonproyong, P. 2012. Improvement of propionic acid production for antifungal activity from whey by calcium alginate immobilization of *Propionibacterium acidipropionici* TISTR 422. Journal of Agricultural Science and Technology. A2: 863-872.

(12) Coelho, L.F., Lima, C.J.B. de, Rodovalho, C.M., Bernardo, M.P. and Contiero, J. 2011. Lactic acid production by new *Lactobacillus plantarum* LMISM6 grown in molasses: optimization of medium composition. Journal of Chemical Engineering. 28(1): 27-36.

(13) Coral, J., Karp, S.G., Vandenberghe, L.P. de S., Parada, J.L., Pandey, A. and Soccol, C.R. 2008. Batch fermentation model of propionic acid production by *Propionibacterium acidipropionici* in different carbon sources. Journal of Appl. Biochem. Biotechnol. 151: 333-341.

(14) Cristian, J., Lima, B. D., Coelho, L.F., Blanco, K.C. and Contiero, J. 2009. Response surface optimization of D(-)-lactic acid production by *Lactobacillus* SMI8 using corn steep liquor and yeast autolysate. Journal of Biotechnology. 8(21): 5842-5846.

(15) Dimova, N.D., Iovkova, Z.S., Brinkova, M. and Godjevargova, Ts. I. 2010. Production of *Candida* biomass from hydrolysed agricultural biowaste. Journal of Biotechnol. and Biotechnol. 24(1): 1577-1581.

(16) Dishisha, T., Alverez, M.T. and Hatti-Kaul, R. 2012. Batch and continuous propionic acid production from glycerol using free and immobilized cells *Propionibacterium acidipropionici*. Journal of Bioresour. Technol. 128C: 679-687.

(17) Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. 1956. Colorimetric method for determination of sugars and related substances. Journal of Analyt. Chem. 28: 350-356.

(18) EI-Deek, A.A., Ghonem, K.M., Hamdy, S.M., Aser, M.A., Aljassas, F.M. and Osman, M.M. 2009. Producing single cell protein from poultry manure and evaluation for broiler chickens diets. Journal of Poultry Science. 8(11): 1062-1077.

(19) Feng, X., Chen, F., Xu, H., Wu, B., Li, H., Li, S. and Ouyang, P. 2011. Green and economical production of propionic acid by *Propionibacterium freudenreichii* CCTCC M207015 in plant fibrous-bed bioreactor. Journal of Bioresource Technology. 102: 6141-6146.

(20) Feng, X., Wu, B., Shen, X. and Xu, H. 2008a. Propionic acid fermentation by *Propionibacterium freudenreichii* CCTCC M207015 with a fibrous-bed bioreactor. Journal of Biotech. 24(6): 1075-1079.

(21) Feng, X., Wu, H., Yao, J., Zhu, H. and Ouyang, P. 2008b. Kinetic analysis and pH-shift control strategy for propionic acid production with *Propionibacterium freudenreichii* CCTCC M207015. Journal of Appl. Biochem. Biotechnol. in press (doi: 10.1007/s 12010-008-8300-6).

(22) Givry, S., Prevot, V. and Duchiron, F. 2008. Lactic acid production from hemicellulosic hydrolysate by cells of *Lactobacillus bifermentans* immobilized in Ca-alginate using response surface methodology. Journal of Microbiol. Biotechnol. 24: 745-752.

(23) Goswami, V. and Srivastava, A.K. 1999. Fed-batch propionic acid production by *Propionibacterium acidipropionici*. Journal of Biochemical. Engineering. 4: 121-128.

(24) Gupta, A. and Srivastava, A.K. 2001. Continuous propionic acid production from cheese whey using in situ spin filter. Journal of Biotechnol. Bioprocess. 6: 1-5.

(25) Himmi, E.H., Bories, A., Boussaid, A. and Hassani, L. 2000. Propionic acid fermentation of glycerol and glucose by *Propionibacterium acidipropionici* and *Propionibacterium freudenreichii* ssp. *shermanii*. Journal of Appl. Microbiol. Biotechnol. 53: 435-440.

(26) Honarato, T.L., Rabelo, M.C., Pinto, G.A.S. and Rodrigues, S. 2007. Produção de ácido lático e dextrana utilizando suco de caju como substrato. Journal of Cienc. Technol. Aliment. 27: 254-258.

(27) Huang, Y.L., Wu, Z., Zhang, L., Cheung, C.M. and Yang, S. 2002. Production of carboxylic acids from hydrolyzed corn meal by immobilized cell fermentation in a fibrous-bed bioreactor. Journal of Bioresour. Technol. 82: 51-59.

(28) Irfan, M., Nazir, M.I., Nadeem, M., Gulsher, M., Syed, Q. and Baig, S. 2011. Optimization of Process parameters for the production of single cell biomass of *Candida utilis* in solid state fermentation. Journal of Agric. and Environ. Sci. 10(2): 264-270.

(29) Jin, Z. and Yang. S.T. 1998. Extractive fermentation for enhanced propionic acid production from lactose by *Propionibacterium acidipropionici*. Journal of Biotechnol. Progr. 14: 457-465.

(30) Kiran, R.R.S., Kohduri, R., Rao, G.H and Modhu, G.M. 2010. Statistical optimization of endo-polygalacturonase production by overproducing mutants of *Aspergillus niger* in solid-ststе fermentation. Journal of Biochem. Tech. 2(2): 154-157.

(31) KoŚmider, A., DroŻdŻyŃska, A., Blaszka, K., Leja, K. and Czacayk, K. 2010. Propionic acid production by *Propionibacterium freudenreichii* ssp. *shermanii*. Using crude glycerol and whey lactose industrial wastes. Journal of Environmental. 19(6): 1249-1253.

(32) Lazaridou, A., Roukas, T., Biliaderis, C.G. and Vaikousi, H. 2002. Characterization of pullulan produced from beet molasses by *Aureobasidium pullulans* in a stirred tank reactor under varying agitation. Journal of Microbial Technology. 31: 122-132.

(33) Lee, B.-K. and Kim, J.K. 2001. Production of *Candida utilis* biomass on molasses in different culture types. Journal of Aquacultural Engineering. 25: 111-124.

(34) Li, K., Feng, X., Wu, B., Zhang, Y. and Xu, H. 2008. PVA-calcium alginate immobilization with *Propionibacterium freudenreichii* NX-4 for propionic acid production. Journal of Nanjing Univ. Tech. (Nat. Sci. Ed.). 30(4): 20-24.

(35) Lima, C.J.B. de, Coelho, L.F., Blanco, K.C. and Contiero, J. 2009. Response surface optimization of D(-)-lactic acid production by *Lactobacillus* SMI8 using corn steep liquor and yeast autolysate an alternative nitrogen source. Journal of Biotechnology. 8(21): 5842-5846.

(36) Lima, D., Coelho, C.J.B., Blanco, L.F. and Contiero, J. 2009. Response surface optimization of D(-)-lactic acid production by *Lactobacillus* SMI8 using corn steep liquor and yeast autolysate as an alternative nitrogen source. Journal of Biotechnol. 8: 5842-5846.

(37) Liu, Y., Zhang, Y.-G., Zhang, R.-B., Zhang, F. and Zhu, J. 2011. Glycerol/glucose co-fermentation: one more proficient process to produce propionic acid by *Propionibacterium acidipropionici*. Journal of Curr. Microbiol. 62: 152-158.

(38) Liu, Z., Ma, C., Gao, C. and Xu, P. 2012. Efficient utilization of hemicellulose hydrolysate for propionic acid production using *Propionibacterium acidipropionici*. Journal of Bioresource Technology (doi: 10.1016/j.biortech.2012.02.118).

(39) Lowery, O.H., Rosier, A.F. and Randall, R. 1951. Protein measurement with folin phrnol reagent. Journal of Biol. Chem. 242: 265-275.

(40) Morales, J., Choi, J. and Kim, D. 2006. Production rate of propionic acid in fermentation of cheese whey with enzyme inhibitors. Journal of Environ. Prog. 25(3): 228-234.

(41) Mumawar, R.A., Irfan, M., Nadeem, M., Syed, Q.A. and Siddique, Z.H. 2010. Biosynthesis of single cell biomass of *Candida utilis* by submerged fermentation. 62(1): 1-5.

(42) Nasseri, A.T. Rasoul-Amini, S., Morowvat, M.H. and Ghasemi, Y. 2011. Single cell protein: production and process. Journal of Food Technology. 6(2): 103-116.

(43) Naveena, B.J., Altaf, Md., Bhadriah, K. and Reddy, G. 2005. Selection of medium components by Plackett-Burman design for production of L(+) lactic acid by *Lactobacillus amylophilus* GV6 in SSF using wheat bran. Journal of Bioresource Technology. 96: 485-490.

(44) Plackett, R.L. and Burman, J.P. 1946. The design of optimal multifactorial experiments. Journal of Biometrika. 33.

(45) Quesadachanto, A., Afschar, A.S. and Wagner, F. 1994a. Optimization of a *Propionibacterium acidipropionici* continuous culture utilizing sucrose. Journal of Appl. Microbiol. Biotechnol. 42: 16-21.

(46) Rajoka, M.I., Khan, S.H., Jabbar, M.A., Awan, M.S. and Hashmi, A.S. 2006. Kinetic of batch single cell protein production from rice polishings with *Candida utilis* in continuously aerated tank reactors. Journal of Bioresource Technology. 97: 1934-1941.

(47) Rajoka, M.I., Kiani, M.A.T., Khan, S., Awan, M.S. and Hashmi, A.-S. 2004. Production of single cell protein from rice polishings using *Candida utilis*. Journal of Microbiology and Biotechnology. 20: 297-301.

(48) Ramsay, J.A., Hassan, M.C.A. and Ramsay, B.A. 1998. Biological conversion of hemicellulose to propionic acid. Journal of Enzyme Microb. Technol. 22: 292-295.

(49) Rosma, A. and Cheong, M.W. 2007. Effect of nitrogen supplementation on yeast (*Candida utilis*) biomass production by using pineapple (*Ananas comosus*) waste extracted medium. Journal of Microbiology. 3(1): 19-26.

(50) Rosma, A. and Ooi, K.I. 2006. Production of *Candida utilis* biomass and intracellular protein content: effect of agitation speed and aeration rate. Journal of Microbiology. 2(2). 15-18.

(51) Rosma, A. Liong, M.T., Mohd. Azemi, M.N. and Wan Nadiah, W.A. 2005. Optimization of single cell protein production by *Candida utilis* using juice extracted from pineapple waste through response surface methodology. Journal of Microbiology. 1(1): 18-24.

(52) Roukas, T. 1998. Pretreatment of beet molasses to increase pullulan production. Journal of Process Biochemistry. 33(8): 805-810.

(53) Shao, N., Wang, D., Wei, G., Zhang, Q., Ge, X. and Nie, M. 2010. Screening of *Candida utilis* and medium optimization for co-production S-adenosylmethionine and glutathione. Journal of Chem. Eng. 27(6): 1847-1851.

(54) Suwannakham, S. and Yang, S.-T. Enhanced propionic acid fermentation by *Propionibacterium acidipropionici* mutant obtained by adaptation in a fibrous-bed bioreactor. Journal of Biotechnol. Bioeng. 91: 325-337.

(55) Villas-BÔas, S.G., Esposito, E. and Margarido, M. de M. 2003. Bioconversion of apple pomace into a nutritionally enriched substrate by *Candida utilis* and *Pleurotus ostreatus*. Journal of Microbiology and Biotechnology. 19: 461-467.

(56) Wood, B.J.B. and Holzapfel, W.H. 1995. The genera of lactic acid bacteria. Glasgow: Blackie Academic and Professional.

(57) Xiao, Z.J., Liu, P.H., Qiu, J.Y. and Xu, P. 2007. Statisticaal optimization of medium components for enhanced acetoin production from molasses and soybean meal hydrolysate. Journal of Appl. Microbiol. Biotechnol. 74: 61-68.

(58) Zhang, A. and Yang, S.-T. 2009. Engineering *Propionibacterium acidipropionici* for enhanced propionic acid tolerance and fermentation. Journal of Biotechnology and Bioengineering. 104(4): 766-773.

(59) Zhang, Y., Wang, Y., Wang, Z.-G., Wang, X., Guo, H.-S., Meng, D.-F. and Wong, P. 2012. Optimization of fermentation medium for the production of atrazine degrading strain Actinetobacter sp. DNS32 by statistical analysis system. Journal of Biomedicine and Biotechnology. 2012, Article ID 623062: 1-7.

(60) Zhao, G. Zhang, W. and Zhang, G. 2010. Production of single cell protein using waste capsicum powder produced during capsanthin extraction. Journal of Applied Microbiology. 50: 187-191.

(61) Zhu, L., Wei, Peilian, Cai, J., Zhu, X., Wang, Z., Huang, L. and Xu, Z. 2012. Improving the productivity of propionic acid with FBB-immobilized cells of an adapted acid-tolerant *Propionibacterium acidipropionici*. Journal of Bioresource Technology. 112: 248-253.

(62) Zhu, Y.F., Li, J., Tan, M., Liu, L., Jiang, L., Sun, J., Lee, P., Du, G. and Chen, J. 2010. Optimization and scale-up propionic acid production by propionic acid-tolerant *Propionibacterium acidipropionici* with glycerol as the carbon source. Journal of Bioresource Technology. 101: 8902-8906.