Cellulase, mannanase and xylanase as novelty mixed enzyme RT-P3 powder through solid-state fermentation from copra meal residue for fed-batch ethanol fermentation

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Abstract—The objective of this study is to investigation of mixed enzyme RT-P3 such as mannanase, xylanase and cellulase from solid-state fermentation from copra meal residue using co-culture of *Trichoderma reesei* and *Saccharomyces cerevisiae* and the application of mixed enzyme RT-P3 powder in batch and fed-batch ethanol fermentation. The suitable condition of fresh mixed enzyme RT-P3 was 100 g copra meal residue and adjusted to 78 %w/w initial moisture content by 300 mL of 3% sugar in liquid medium pH 5 with included initial co-culture microorganism of 7.07×10^5 cell/mL and incubated at 24±2°C for 6 days. After that this mixed enzyme was dried at 65°C in a dryer to obtain powder at 7% w/w moisture content before used for ethanol fermentation through consolidate bioprocessing. It was found that the maximum ethanol was 33.0 and 47.7 g/L of batch and fed-batch fermentation respectively at 4 days incubation and room temperature (30°C).

Keywords - cellulase, mannanase, xylanase, copra meal residue, ethanol

I. INTRODUCTION

Lignocellulosic materials is composed of cellulose, hemi-cellulose, pectin, protein and lignin. The rigid outermost shell of plant is lignin. The inside most compost of cellulose, hemi-cellulose and pectin which has crystalline and, or amorphous region. Amorphus cellulose can be easily degraded more than crystalline cellulose. Due to cellulose is contained of glucose chain molecule with β-1,4-linkages between glucose and hydrogen bond in amorphous region. Hemi-cellulose has a polymer of xylan, mannan, galactan and arabinan. Xylan polymer is the most major component in the nature which bounded hydrogen bond with cellulose as layer and covalent bond with lignin. Mannan is a major component of hemi-cellulose as well which contained of mannose, galactose and glucose in structure molecule. Pectin is hydrophilic polysaccharide which major composition is galacturonic acid [1]. Thus lignocellulosic materials has potential utilization for ethanol production because of these can be converted to glucose by several methods such as diluted and concentrated acid hydrolysis, enzymatic hydrolysis, gamma-ray or electron-beam irradiation and microwave [2].

Copra meal residue (CMR) is a solid by-product from coconut milk or copra meal extracted oil. This is used for animal feed or value added for enzyme production. Previous study used coconut oil cake or defatted copra as raw material for enhanced mannanase production by submerged culture fermentation of *Aspergillus niger* NCH-189. It was found that CMR is the best carbon source for mannanase production which contain nitrogen and mannan component. The result was 28 U/mL mannanase activity for 3 day fermentation at 30°C [3]. Beside *Trichoderma harzianum* strain T4 was used to produce mannanase in liquid state fermentation with wheat bran as carbon source. The maximum mannanase activity was found at 6-8 day fermentation about 8.5 IU/mL [4].

Another previous study of mannanase production using recombinant *Aspergillus sojae* ATCC11906 (AsT1). It was found that the highest β-mannanase activity was 363U/ml on the fourth day of cultivation at 30°C in the optimized medium consisting of 7% sugar beet molasses, 0.43% NH_4NO_3, 0.1% K_2HPO_4 and 0.05% $MgSO_4$ (w/v) at 207rpm. On the sixth day of cultivation under the optimized conditions, the highest β-mannanase activity was achieved as 482U/ml which is 1.4-fold of 352U/ml activity found on glucose medium previously [5].

Enzymatic hydrolysis can be used for degradation of cellulose and hemi-cellulose in to glucose by using suitable commercial enzymes and microorganisms such as *Bacillus* sp., *Clostridium* sp., *Chaetomium* sp. and *Aspergillus* sp. [6]. Then *Saccharomyces cerevisiae* can convert glucose to ethanol. Moreover commercial enzymes, such as cellulase (Spezyme CP) derived from *T. reesei*, xylanase (Multifect Xylanse) derived

from genetically modified strain of *T. reesei*, pectinase (Multifect Pectinase) derived from a selected strain of *A. niger* and β-glucosidase (Novozyme 188), were performed as enzyme complexes for enzymatic hydrolysis of lignocellulose to enhance the conversion of cellulose and hemicellulose from corncob pretreated by ammonia soking [7]. From the previous literature, lignocellulosic materials must be pretreated by some method. Although conventional process of ethanol production from lignocellulosic biomass includes four main steps such as pretreatment, enzymatic hydrolysis, fermentation and distillation-rectification-dehydration. The new improvements was reported [8]. However this study will avoid of environmental impact from pretreatment process. Therefore CMR was directly used for feasibility mixed enzyme RT-P3 powder production without pretreatment.

Previous study of ethanol production has developed consolidated bioprocess (CBP) of lignocellulosic materials with thermotolerant yeast strains engineered where enzyme production, saccharification and fermentation are all performed in one unit operation compared with simultaneous saccharification and fermentation (SSF) in order to reduce the investment of capital cost, substance and other raw materials and utilities. In this review reported that in SSF process, thermotolerant ethanologenic yeast strain such as *Kluyveromyces maxianus*, can convert steam explosion pretreated of lignocellulosic materials such as poplar, sweet sorghum, bagasse and wheat straw, to ethanol via SSF process at 42°C. Ethanol was 16-19 g/L and 50-72% yield after 72-72 h fermentation. *K. maxianus* strain was used to produce 22.5 g/L ethanol from 80 g/L cellulosic solid from hydrothermal pretreated of switchgrass in batch after 168 h at 45°C which was 86% yield. Moreover *Pichia kudriavzevii* (*Issactachenkia orientalis*) IPE100 was applied to the fermentation of enzymatic hydrolysate of steam-exploded cornstalks at 42°C which ethanol yield was 93.8%. And also sugarcane juice containing of 140 g/L sucrose, 15 g/L glucose and 9 g/L fructose were fermented with *P. kudriavzevii* to produce 71.9 g/L ethanol at 40°C. Moreover newly isolated *P. kudriavzevii* was used to produce 35% more ethanol at 40°C from 150 g/L glucose and more at 45°C than conventional *S. cerevisiae* [9].

Addition to the above review study of CBP reported that using recombinant strain (*K. maxianus* NBRC1777, *Aspergillus niger* and *Thermoascu aurantiacus*) can produce 43.4 g/L ethanol from 100 g/L cellobiose. The recombinant *K. maxianus* strain was engineered with *Trichoderma reesei* and *A. aculeatus* to enhance cellulose conversion efficiency which produced 20.4 g/L ethanol from 53.4 g/L cellobiose at 45°C. Although thermotolerant and ethanologenic strains were efficiently used for SSF of lignocellulosic biomass at high temperature. However construction of recombinant strains has been limited to yeast species used such as *K. maxianus* and *P. kudriavzevii* due to lack of effective genetic tools. One of the key factor of CBP are necessary to pretreat biomass and use recombinant strains or mixed enzymes which are suitable for the advanced industrial technology's countries. Therefore this study will focus on solid-state and submerge fermentation from copra meal residue with the native co-culture microorganisms of *Trichoderma reesei* and *Saccharomyces cerevisiae* which can provide mixed enzymes RT-P3 such as mannanase, xylanase and cellulase for direct conversion of lignocellulosic material to ethanol through CBP without biomass pretreatment

T. reesei and *S. cerevisiae* have been natural cultured in the same potato dextrose agar plate to use for cellulase, mannanase and xylanase production under solid state fermentation in this study. CMR is used as substrate without any pretreatment. From the review of fungal β-mannanases: mannan hydrolysis, heterologous production and biotechnological applications of [10] had been reported the variety applications of β-mannanases in nutraceutical production (production of mamo-oligosaccharide; pharmaceutical), food and feed (hydrolysis of mannan in spent coffee ground; animal feed in monogastric animal) and commodity production (biofuel, oil and gas well stimulation; paper and pulp and detergent formulation). Due to the requirement of cellulase, mannanase and xylanase in several fields then this study will focus on type of liquid medium that suitable for enzymes production and also the application of these enzyme for CPB of ethanol production from CMR.

II. RESEARCH METHODOLOGY

A. Biomass feedstock

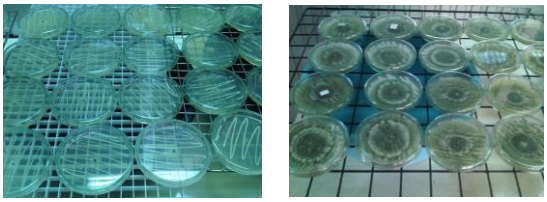
Copra meal residue (CMR) was obtained from the market near Rajamangala University of Technology Thanyaburi, Klong 6, Thanyaburi, Pathumtani 12110. Thailand. The dehydration residue has moisture content less than 13%w/w after sun-dried. Average particle size of 1.94 mm of CMR was dried. This dried substrate was chemical composition analyzed, measured moisture content, sterilized in autoclave at 120°C and 14.7 psig for 20 min, and then cooled at room temperature prior used for solid-state and submerge fermentation.

B. Types of Liquid medium

The types of liquid medium were LM (2g/L as N), LMY (3g/L as N), LMP (7g/L as N), and LMYP (8g/L as N), which each compost of about 8 g/L urea, 8 g/L urea plus 10 g/L yeast extract, 8 g/L urea plus 30 g/L peptone, and 8 g/L urea plus 10 g/L yeast extract plus 30 g/L peptone as nitrogen source respectively. Additional ingredient of each liquid medium was composed of 15 g phosphate-potassium fertilizer; NPK-0-52-34, 1 g calcium hydrogen phosphate 2 hydrate; $CaHPO_4 \cdot 2H_2O$, 1 g magnesium sulfate 7 hydrate; $MgSO_4 \cdot 7H_2O$, 30 g sugar and 1 L pure water. These liquid medium was adjusted to pH 5 and sterilized in autoclave at 14.7 psig and 120°C for 20 min. Then cooled at room temperature before used.

C. Co-culture microorganism RT-P3 preparation

The co-culturing of *T. reesei* RT-P1 and *S. cerevisiae* RT-P2 was carried out by inoculating both strains onto potato dextrose agar (PDA) in a same Petri dish. The process began with streaking *S. cerevisiae* RT-P2 on PDA using a loop needle. *T. reesei* RT-P1 from a PDA slant was then transferred by infecting a needle onto the center of the Petri dish, as shown in Fig.1 (a). The incubation period was 7 days at room temperature (30°C). Fig.1 (b) illustrates the co-cultured microorganisms after the 7 day incubation period which is defined as co-culture microorganism RT-P3.



(a) (b)

Figure 1 Co-culture microorganism on PDA plate (a) Initial incubation (b) 7 days incubation

D. CMR solid-state fermentation with co-culture microorganism RT-P3 in various types of liquid medium

The first step, co-culture microorganism RT-P3 from surface of 2 PDA plates was inoculated into 1 L of each type of sterilized liquid medium i.e. LM, LMY, LMP and LMYP respectively. The initial microorganism concentration in each various LM after mixed by magnetic stirred for 30 minutes was averaged of 7.22×10^5 cell/mL. The second step, solid-state fermentation was performed in 1 L glass bowl to investigate the optimal condition of nitrogen source in various type of liquid medium for CMR under solid-state fermentation by using 50 g fixed sterilized CMR, and then mixing a substrate with 50, 100, 150 and 200 mL of each type of liquid medium at pH 5. Then each bowl was covered with perforated rape film before incubated at 24±2°C for 7 days. The sample was collected every day to measure cellulase, mannanase, xylanase activity, reducing sugar and cell concentration from initial to 7 days incubation. All data was done in duplicates. Finally, once the optimal type of liquid medium and the ratio of CMR to liquid medium volume (i.e. 1:3w/v of CMR:LM for 6 days incubation at 24±2°C) was identified, the production of a larger-scale mixed enzymes RT-P3 powder was carried out in 10L polypropylene (PP) containers under the optimal condition.

E. Mixed enzyme RT-P3 powder production from CMR

The 250 g sterilized copra meal residue was investigated in 10 L clear PP box under solid-state fermentation at the optimal condition of liquid medium type and the ratio of CMR to liquid medium volume which contained initial microorganism concentration averaged of 7.22×10^5 cell/mL. Liquid medium, LM at pH 5 was extended 2.5 times volume of shaking flask level in order to adjust moisture content. The product obtained was defined as fresh mixed enzyme RT-P3. Then it was dehydrated and pulverized to perform mixed enzyme RT-P3 powder.

Figure 2 (a) Dried CMR (b) CMR fermentation in glasses bowl with co-culturing of *T. reesei* and *S. cerevisiae* (c) CMR cultivation in 10 L PP boxes in the incubator

F. Orthogonal experimental method for ethanol batch fermentation

The ethanol submerged batch fermentation was investigated in 250 mL shaking Erlenmeyer flask at 100 rpm for 24 hours per day. Total 25 runs was perform using orthogonal experimental method by varied 5 level of CMR weight (2, 4, 6, 8, 10 g), mixed enzyme RT-P 3 powder weight (1, 2, 3, 4, 5 g), sugar concentration in 100 mL of LM (0, 1, 2, 3, 4 g) and incubation time (1, 3, 5, 7, 9 day). Ethanol concentration in hydrolysate was analyzed in order to find the optimal condition of this batch submerged fermentation.

G Ethanol batch and fed batch submerged fermentation

A ethanol batch fermentation was operated in 15 L cylindrical glass tank and controlled volume of 3 L at the optimal condition obtained from orthogonal experimental method (2.6). However the ethanol batch fermentation was extended to 30 times from shaking flask level. This process was aerated and mixed with a paddle at 200 rpm for 6 hours per day. Ethanol, reducing sugar and cell concentration of hydrolysate were analyzed every day. A fed batch fermentation was investigated as same as batch fermentation except for feeding new liquid medium (3% sugar) of 500 mL into the fermentation tank at the second day and every day until 7 days incubation. Ethanol, reducing sugar and cell concentration of hydrolysate were analyzed every day.

H. Analytical method

The chemical composition analysis of CMR was investigated base on TAPPI 203 om-88 [11] for cellulose and hemi cellulose, TAPPI T211 om-02 [12] for ash and TAPPI 222 om-88 [13] for lignin and Soxhlet extraction for fat content at 6% moisture content (dry basis).

The concentration of microorganism was analyzed from the initial and every day of fermentation. The sample was prepared using 0.5 g mixed enzyme RT-P3 powder in 10 mL pure water. Then the suspended solid was mixed with vortex mixer before determined using a haemacytometer (Boeco, Germany) with 40X microscope.

Mannanase activity was assayed by mixing 0.5 mL of diluted enzyme solution with 0.5 mL of 0.5% locust bean gum in 50 mM citric-citrate buffer (pH 4) at 60°C for 30 min [14]. The reducing sugar released was determined by DNS method using mannose as standard [15]. One unit (IU) of enzyme activity is defined as the amount of enzyme required producing 1 micromole of mannose per minute under the assay condition.

Xylanase activity was determined as reported elsewhere using Birchwood xylan (Sigma) as a substrate [16]. The reducing sugar after 10-min incubation at 50°C was measured by 3, 5-dinitrosalicylic acid (DNS) reagent. One unit (IU) of enzyme activity is defined as the amount of enzyme required to liberate 1 micromole of glucose per minute at pH 5 and at 50°C.

Cellulase activity was determined with Whatman No. 1 filter paper [17]. The reducing sugar after 60-min incubation at 50°C was measured by 3, 5-dinitrosalicylic acid (DNS) reagent. One unit (IU) of enzyme activity is defined as the amount of enzyme required to liberate 1 micromole of glucose per minute at 50°C.

Reducing sugar concentration was determined by DNS method. Ethanol concentration was analyzed by fresh distillation method [18].

III. RESULTS & DISCUSSION

A. Chemical composition of CMR

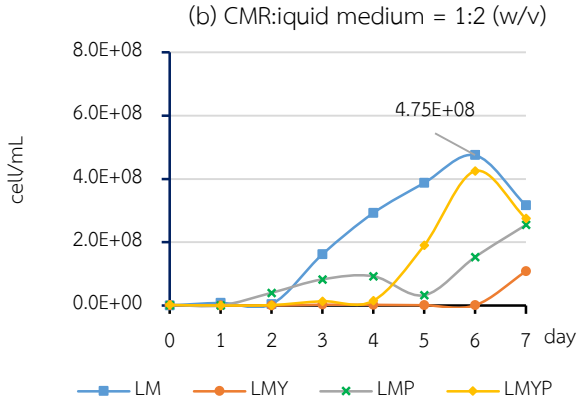
The result of chemical composition of CMR was 48.7% cellulose, 0.4% hemi cellulose, 31.2% lignin, 19.3% oil and 0.4% ash by weight. Percent of lignin in CMR is very high due to this raw materials has brown color of thin layer seed coat which is lignin mixed with white color coconut meal as shown in Fig. 2 (a). For Fig 2 (b) and (c) show the co-culture *T. reesei* and *S. cerevisiae* can be grown on CMR in glasses bowl after 6 day fermentation and 10 L of PP boxes in the incubator, though CMR has high lignin content.

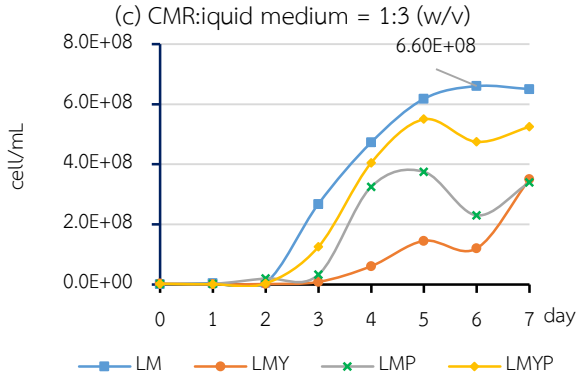
A few microorganisms are able to degrade lignin, including bacteria such as *Steptomyces* sp. and *Nocardia* sp. and basidiomycetes (brown-rot and white-rot fungi, respectively). The natural microbial decay system are effective but slow [19]. Beside lignin, hemicellulose content and biomass structure i.e. particle size and porosity another factors affecting cellulose accessibility for enhancing ethanol production from lignocellulosic biomass are surface area of cellulose. This study used native microorganism between *T. reesie* RT-P1 which can degrade cellulose into monosaccharide and cellobiose and then *S. cerevisiae* RT-P2 converted sugar to ethanol simultaneously. There was some lignin mixed in CMR as depicted in Fig. 2 (a) but the co-culture microorganism could be grown on CMR as shown in Fig. 2 (b) and (c) respectively. Due to the previous study had reported that composition of CMR which was analyzed using AOAC method contained 79.77% mannose, 12.80% glucose, 6.12% galactose and 1.31% arabinose [20].

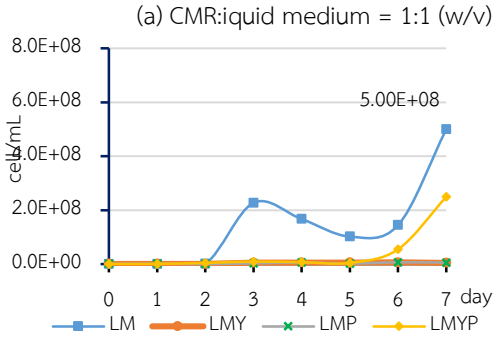
B. Effect of type and initial volume of liquid medium on mixed enzymes RT-P3

The 4 types of liquid medium were investigated for mixed enzymes RT-P3 production from CMR under solid-state fermentation with co-culture microorganism of *T. reesie* and *S. cerevisiae*. It was found that LM provided microorganism concentration higher than LMY, LMP and LMYP at the ratio of CMR to liquid medium volume of 1:1, 1:2, 1:3 and 1:4 at 6 days incubation as shown in Fig. 3 (a) – (d) respectively. The maximum cell growth was 6.60E+8 cell/mL at 1:3 as shown in Fig. 3 (c). Even though LMYP was suitable liquid medium for cultivation yeast strain. However the co-culture microorganism

was mixed of two strains between fungi and yeast. Moreover effect of initial liquid medium volume of LMY and LMP on cell growth was more than 3 day lag phase. Therefore the suitable of liquid medium type was LM which was 2 days lag phase. Due to this study similar the previous studied which has been reported the relation between xylanase activity and cell growth or biomass production under solid-state fermentation of dried citrus peel employing *A. niger F3*. Enzyme activity was maximum at incubation time of the highest biomass obtained[6].







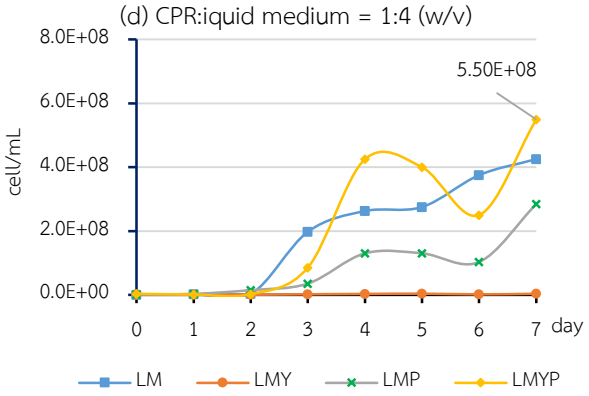


Figure 3. Cell growth profile with time of CMR under solid-state fermentation for various types of liquid medium

Therefore 6 and 7 days incubation of solid-state fermentation were fixed in order to compare type of liquid medium and the ratio of CMR to liquid medium volume which provided the highest cellulase, mannanase and xylanase activity as shown in Fig. 4, 5 and 6 respectively. It was found that LM at 1:3 (w/v) CMR:LM was the optimal condition of mixed enzyme RT-P3 production which the maximum of cellulase, mannanase and xylanase activity were 50.9, 16.1 and 10.9 IU/gds at 6 days incubation and 24±2°C, respectively.

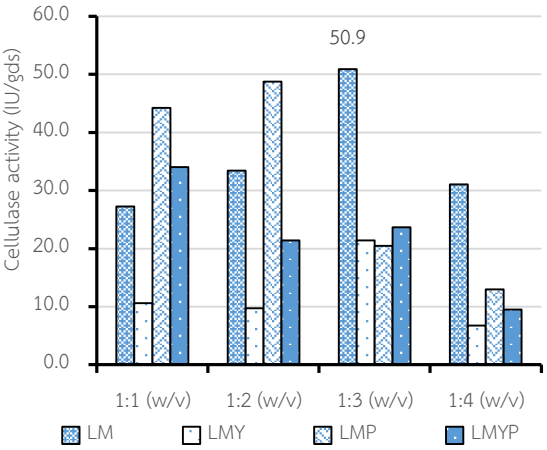


Figure 4 Cellulase activity of solid-state fermentation from CMR with co-culture microorganism in various types of liquid medium at 6 and 7 days incubation using the ratio of CMR:liquid medium volume of 1:2 (w/v), 1:3 (w/v) and 1:1 (w/v) and 1:4 (w/v), respectively

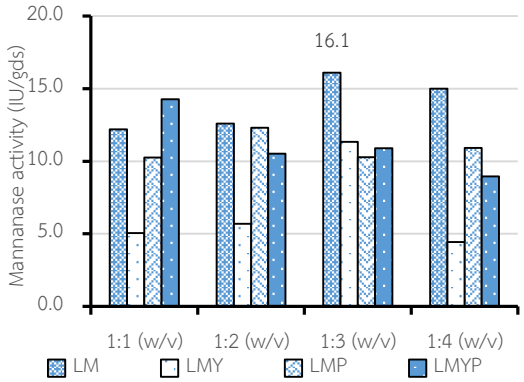


Figure 5 Mannanase activity of solid-state fermentation from CMR with co-culture microorganism in various types of liquid medium at 6 and 7 days incubation using the ratio of CMR:liquid medium volume of 1:2 (w/v), 1:3 (w/v) and 1:1 (w/v) and 1:4 (w/v), respectively

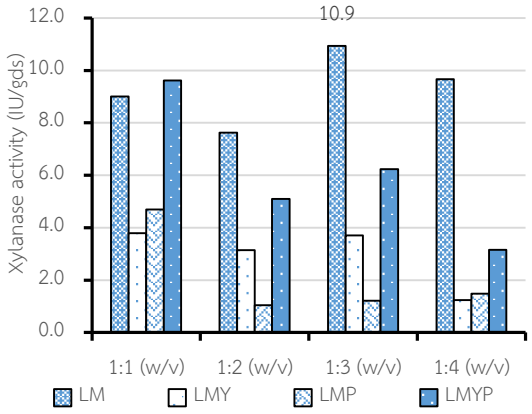


Figure 6 Xylanase activity of solid-state fermentation from CMR with co-culture microorganism in various types of liquid medium at 6 and 7 days incubation using the ratio of CMR:liquid medium volume of 1:2 (w/v), 1:3 (w/v) and 1:1 (w/v) and 1:4 (w/v), respectively

From the previous studied has been reported effect of nitrogen addition on enzyme activity of individual fungi such as *Penicillium*, *Aspergillus* and *Trichoderma*. It was found that more nitrogen addition to *Trichoderma* less obtained enzyme activity [21]. Cellulase, mannanase and xylanase activity might be inhibited by excess nitrogen in liquid medium for this study. Therefore mixed enzyme production should be carried on using LM which compost of 8 g/L urea as 2g/L-N source and 6 days of incubation at 24±2°C and pH5. The application of LM for these fermentation can reduce cost of liquid medium due to it is not necessary to use expensive chemicals supplementary i.e. yeast extract and peptone. Although several previous studies of cellulas, mannanase and xylanase production, the first of the modified liquid medium was investigated for enhanced mannanase production by recombinant *A. sojae* which were molasses and NH_4NO_3 [5]. The second was investigated type of liquid medium to improve mannanase production by *A. niger* which was bacteriological peptone [22]. Therefore type of suitable medium depended on microorganism strain. And also cellulase and xylanase were found to be dependent upon the nature of carbon source and nutrients used in culture media [23].

For the ratio at 1:1 and 1:2 of 4 liquid medium types in this study were insufficient of initial moisture content due to poor accessibility of nutrients. And also at 1:4 was excess of initial moisture content causing low substrate porosity and thus reducing oxygen penetration [24-25]. Furthermore, lower moisture content caused reduction in nutrient solubility, low degree of swelling, and high water tension [26].

The study of enzymes production can be produced from carbon and nitrogen source under solid-state fermentation by suitable microorganism strain, liquid medium, initial moisture content, pH and temperature as this study [27-28]. Moreover a novel fed batch strategy in the next should be applied for industrial fermentation by mixed enzyme RT-P3 powder study for development.

C. Mixed enzyme RT-P3 powder production

The solid state fermentation was extended to 2.5 time of the optimal condition obtained from 3.2 for mixed enzyme RT-P3 powder production. Then it was performed using 250g CMR in 750 mL LM which contained initial microorganism concentration averaged of 7.22×10^5 cell/mL at pH 5 and 24±2°C for 6 days incubation.

It was found that the average cellulase, mannanase and xylanase activity of fresh mixed enzyme RT-P3 were 82.7, 20.5 and 14.8 IU/gds, respectively as shown in Fig. 7. Therefore enzymes activity were 62.5, 27.3 and 35.8% increased from laboratory scale. The powder of mixed enzyme RT-P3 was performed by dehydration fresh mixed enzyme RT-P3 at 65°C for 3 days in the oven and then pulverized. It was found that cellulase, mannanase and xylanase activity of this powder was 27, 44 and 22% decreased. This indicated that some of two strains were died by high temperature. Enzyme activity of mixed enzyme RT-P3 powder was less than the previous research due to *S. cerevisiae* RT-P2 could not efficiently degrade cellobiose to glucose [29]. However the powder of mixed enzyme RT-P3 was further used in ethanol batch and fed batch submerged fermentation [30].

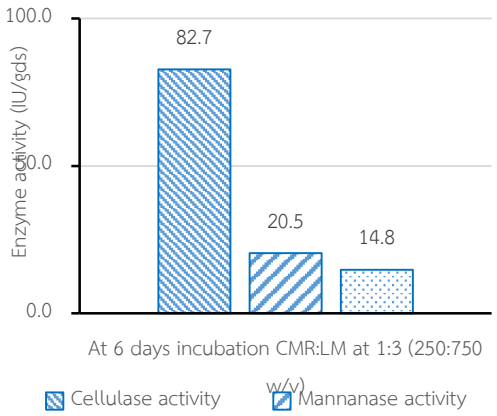


Figure 7 Enzyme activity of fresh mixed enzyme RT-P3 for solid state fermentation at the ratio CMR to LM of 1:3 w/v at pH 5 and 24±2°C for 6 days incubation

D. Ethanol batch fermentation using mixed enzyme RT-P3 powder

The result of using mixed enzyme RT-P3 powder to produce ethanol batch fermentation from CMR in LM was as following,

D.1 The result from orthogonal experimental method of batch fermentation

The orthogonal experimental method was applied to find the optimal condition for ethanol batch fermentation which contained 25 runs in 250 mL Erlenmeyer flask. The result was represented as Table 1. Data from each parameter was calculated to find obtained ethanol concentration as shown in Table 2. It was found that the maximum ethanol concentration was obtained when used 10g CMR, 3g sucrose, 5 days incubation and 4 g mixed enzyme RT-P3 powder in submerged batch fermentation.

TABLE 1 Ethanol concentration of 25 runs batch fermentation result for variation of CMR, enzyme powder, sucrose and fermentation time

Run	CMR, g	Sucrose, g	Time, d	Mixed enzyme RT-P3 powder, g	Ethanol, g/L
1	2	0	1	1	16.26
2	4	1	3	1	45.45
3	6	2	5	1	81.97
4	8	3	7	1	112.96
5	10	4	9	1	76.22
6	2	4	9	2	54.20
7	4	0	1	2	35.32
8	6	1	3	2	65.57
9	8	2	5	2	98.37
10	10	3	7	2	142.09
11	2	3	7	3	133.16
12	4	4	9	3	103.98
13	6	0	1	3	48.21
14	8	1	3	3	86.03
15	10	2	5	3	108.58
16	2	2	5	4	123.21
17	4	3	7	4	152.92
18	6	4	9	4	114.64
19	8	0	1	4	74.19
20	10	1	3	4	111.37
21	2	1	3	5	115.92
22	4	2	5	5	113.93
23	6	3	7	5	148.15
24	8	4	9	5	96.86
25	10	0	1	5	71.34

TABLE 2 The effect of various parameter on obtained ethanol concentration from calculation of Table 1

Parameter	Condition	Ethanol (g/L)
CMR	2 g	75.62
	4 g	73.21
	6 g	77.59
	8 g	74.22
	10 g	**86.85**
Sucrose	0 g	28.18
	1 g	77.90
	2 g	77.54
	3 g	**90.97**
	4 g	88.14
Time	1 d	28.18
	3 d	77.90
	5 d	**102.32**
	7 d	90.97
	9 d	88.14
Mixed enzyme RT-P3 powder	1 g	58.12
	2 g	67.53
	3 g	75.02
	4 g	**97.79**
	5 g	87.77

D.2 The result of ethanol batch and fed batch fermentation in 15L reactor at the optimal condition

The batch fermentation was investigated by extended volume of LM from 100 mL at pH5 in shaking flask to 3000 mL in 15L glass reactor with 6 hours aerated and paddle operated for mixing (120 rpm) at room temperature (30°C). The optimal parameter of CMR, sucrose and mixed enzyme RT-P3 powder were also extended to 300g, 90g and 120g, respectively. However LM was adsorbed by CMR at this condition, this course sample collection difficultly due to reduction of LM volume occurred. Therefore LM volume was 50% increased to avoid this problem. Then used LM volume was 4500 mL.

This experiment has also preliminary investigated for enhance ethanol concentration by using the strategy of fed batch fermentation. Thus new LM of 500 mL was addition fed into reactor at the second day and every day of fermentation.

The maximum ethanol from mixed enzyme powder RT-P3 in batch and fed batch fermentation was 33g/L and 48 g/L at day 4 incubation. This result can be addressed that ethanol production was performed without pretreatment to remove lignin. As ethanol concentration from two-step simultaneous saccharification and co-fermentation using ammonia fiber expansion-treated switchgrass by commercial enzymes and *S. cerevisiae* 424A (LNH-ST) was 36.4 g/L [31]. Addition previous study was ethanol production from pretreated corncob with acid and alkali to remove lignin and using fed-batch simultaneous saccharification and fermentation which the maximum ethanol concentration was 69.2 g/L. Although ethanol obtained from this study was less than the previous study. However the mixed enzyme RT-P3 application was eco-friendly due to non-pretreatment required thus no wastewater to be treatment. And also time used was less.

The maximum average reducing sugar was 76 g/L at day 1 incubation due to the hydrolysis result of mixed enzyme activity all both processes. However reducing sugar was reduced rapidly in fed batch more than in batch fermentation due to addition of new liquid medium which had 3% fresh sugar every day from day 2 until day 5 incubation. Then ethanol obtained from fed batch was higher than batch fermentation significantly, as shown in Fig. 8.

CBP or consolidate bioprocessing was investigated in this study without pretreatment of CMR to remove lignin, simultaneous saccharification and fermentation were performed to produce ethanol by co-culture microorganism from mixed enzyme RT-P3 powder in LM. Cell growth of co-culture microorganism concentration in batch was higher than fed batch fermentation due to dilution of the addition volume of new LM into reactor every day. They were inhibited by lignin in CMR and ethanol concentration, as shown in Fig. 9 and Fig. 10 respectively. In this study there was 23g/L ethanol at initial day for both fermentation due to this ethanol obtained from CMR solid state fermentation with mixed enzyme RT-P3 powder in LM. Although the review reported that ethanol production by CBP at elevated temperature which employed various pretreated lignocellulosic materials and specific thermotolerant or ethanogenic yeast or recombinant strain such as *K. marxinanus* [33-33, 9]. And also obtained ethanol from pretreated corncob using a mutant strain i.e. *Penicillium decumbens* in fed-batch fermentation was 57.2g/L or 5.7% (w/v) at 142 hours [34]. However this study employed non-pretreated CMR and the novel mixed enzyme RT-P3 as the native co-culture strains which could provide about 3% and 5% (w/v) ethanol in batch and fed-batch fermentation.

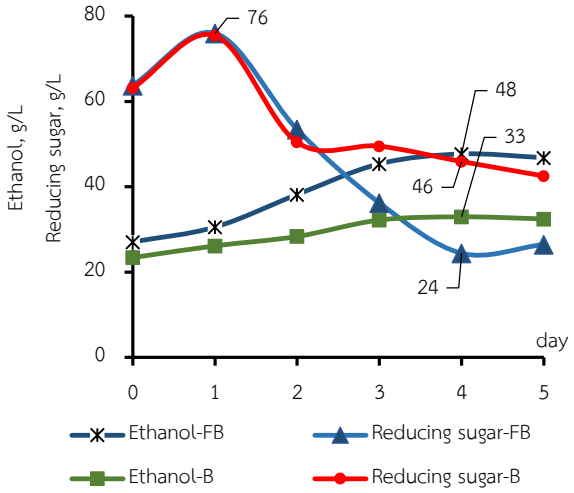


Figure 8 Ethanol and reducing sugar concentration profile with time in 15L glass reactor of batch (B) and fed batch (FB) fermentation

Effect of the new liquid medium addition every day into fed batch fermentation on cellulase, mannanase and xylanase activity was different insignificantly with respect to batch fermentation. It was found that *T. reesie* could degrade cellulose to glucose within 1 day fermentation then activity rapidly reduced at the second day due to both strains microorganism were adsorbed thus these enzymes stay bound to solid and desorb back to the liquid phase to reach equilibrium [35].

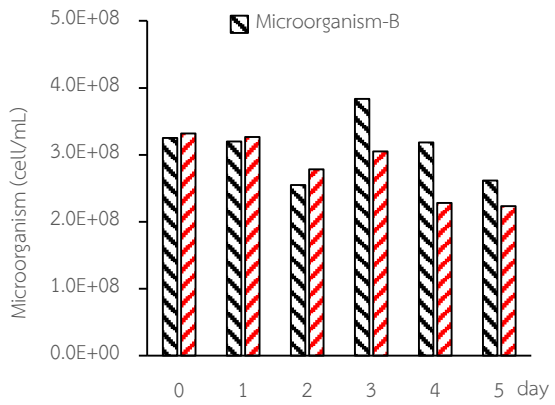


Figure 9 Microorganism concentration with time in 15L glass reactor of batch (B) and fed batch (FB) fermentation

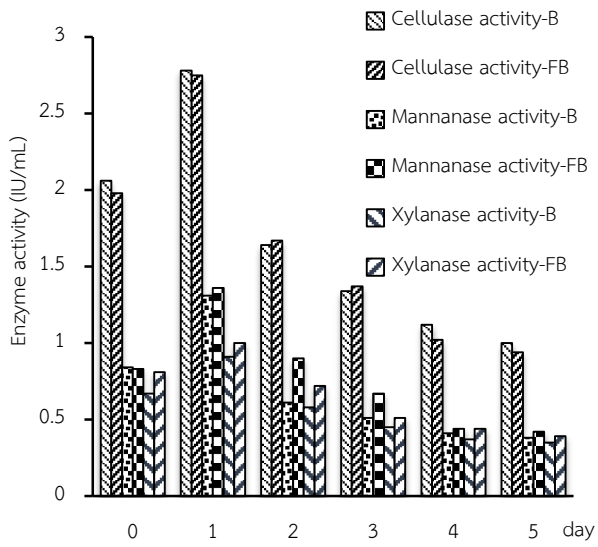


Figure 10 Cellulase, mannanase and xylanase acitvity with time in 15L glass reactor of batch (B) and fed batch fermentation (FB)

IV. CONCLUSION

CMR is lignocellulosic materials which was high potential for mixed enzyme RT-P3 production. Mixed enzyme RT-P3 were cellulase, mannanase and xylanase which were produced under solid state fermentation. Liquid medium is LM that contained N source from commercial urea which is very cheap. The optimal condition of solid state fermentation from CMR with co-culture microorganism of *T. reesei* RT-P1 and *S. cerevisiae* RT-P2 in LM is 250g CMR, 750mL LM at pH 5 and 24±2°C, 6 day incubation and initial microorganism concentration averaged of 7.22×10^5 cell/mL. The average cellulase, mannanase and xylanase activity of fresh mixed enzyme RT-P3 were 82.7, 20.5 and 14.8 IU/gds, respectively. However the powder of mixed enzyme RT-P3 was 27, 44 and 22% decreased due to co-culture microorganism died at 65°C of dehydration.

Ethanol fed batch fermentation is performed in 15L reactor, fed a new LM in the second day and every day until 5 days incubation. Both batch and fed batch submerged fermentation is contained 300g CMR with 120g mixed enzyme RT-P3 powder in LM of 4500 mL, and 6 h/d for aeration and mixing with paddle at 100 rpm. Obtained ethanol is 48g/L which is 94% much more than batch fermentation. Mixed enzyme RT-P3 powder from co-culture between *T. reesei* RT-P1 and *S. cerevisiae* RT-P2 of this study can provide cellulase, mannanase and xylanase activity compared with the previous study of [36] which used mixed cultures of *T. reesei* RUT-C30 and *A. niger* LMA for enhanced enzyme production in fed batch fermentation.

This fed batch fermentation was preliminary studied to produce ethanol from CMR without pretreatment in fed batch fermentation through consolidate biological process. Therefore 500 mL of new LM and the second day of fermentation had been selected from batch fermentation by observation. The result of ethanol from fed batch fermentation was higher than batch fermentation of about 45.5% (w/v). The advantage of this process is environmental friendly due to no wastewater discharged and reduced energy from CMR pretreatment. The native microorganism such as *T. reesei* and *S. cerevisiae* can be cultured on substrate as CMR to produce in the form of powder. This powder as mixed enzyme RT-P3 can be directly used to produce ethanol with LM in fed batch fermentation.

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