Crude Cllulase Powder Production by Solid-State Fermentation Using Cassava Residue and Co-cultured Microorganisms *Trichoderma reesei* and *Saccharomyces cerevisiae*

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- We produced a cheap crude cellulase powder from industrial agriculture solid waste.
- Two local strains, *Trichoderma reesei* and *Saccharomyces cerevisiae*, were mixed in the same incubation. *T. reesei* were used for cellulase production and *S. cerevisiae* for ethanol conversion.
- Crude cellulase powder can be used for digest lignocellulosic materials .
- Crude cellulase powder production is reduced wastewater due to less chemicals used for pretreatment.

Abstract— This research deals with the production of fresh crude cellulase by solid-state fermentation (SSF) using cassava residue and a co-culture of *Trichoderma reesei* and *Saccharomyces cerevisiae* under cassava residue weight, liquid medium volume. The fresh product from SSF was performed for moisture removal until crude cellulase obtained after grinding. Submerged fermentation was investigated using lignocellulosic materials as a substrate suspended in cultivation medium and then mixing with cellulase powder. In the production of fresh crude cellulase, the experiment began of 100 g cassava residue, moisture content 12% w/w and the co-cultured microorganisms in pH 5 liquid medium (LM) of 10^7 cell/mL. To find the suitable moisture content for SSF, 100 g cassava residue, moisture content 12% w/w to LM was tested at ratios of 1:0.6, 1:0.8 and 1:1 w/v, initial sugar concentration in LM was varied between 0.8, 1.6, 2.4 and 3.2%w/w and initial cell concentration is constant. The solid-state fermentation was carried out at $\mathbf{24 \pm 2}^{\circ}$C for 7 days. The optimal condition of SSF are 3.2% w/w initial sugar concentration in 100 mL LM, 100g cassava residue and 6 days of incubation at $\mathbf{24 \pm 2}^{\circ}$C. The fresh crude cellulase with 55% w/w moisture content was subsequently dehydrated and pulverized to produce crude cellulase powder with 12% w/w. The powder was applied to dried pineapple peels fermentation for ethanol production.

Keywords **-** cellulase, solid-state fermentation, ethanol, cassava residue, yeast, filamentous fungi

I. INTRODUCTION

Cassava residue is a lignocellulosic solid waste from the production of tapioca flour and is available in great quantity and low cost in Thailand. Therefore cassava residue is excellent substrate used for bioethanol fermentation due to US$ 9.00/metric ton wet weight and US$ 99.00/metric ton dry weight current prices of cassava residue in Thailand. Because of commercial enzyme is expensive thus for alternative biofuel production require noncommercial enzyme which can be used for bioethanol fermentation in Thailand. And also pretreatment step is skip for reducing cost of bioethanol production.

Lignocellulosic-based bioethanol can be produced by solid-state fermentation using cellulose as substrate cultivated with microorganisms to produce cellulase for glucose conversion and then subsequence by submerged fermentation to produce bioethanol. In order to obtain high yield bioethanol, it necessary to improve local microorganisms that can convert lignocellulosic materials into glucose and simultaneously ethanol without any pretreatment. Due to pretreatment lignocellulosic materials required a lot quantity of energy, chemicals and water which concern to environmental impact. Although many researches that study of pretreatment processes to increase efficiency of fermentation for example, the pretreatment by biological conversion of feedstock [1], the pretreatment of corn fibers with mild alkali and steam [2], rice hulls with lime [3], maize straw with 2%NaOH at 80°C for 1 hr [4] and of rapeseed straw with H_2SO_4-catalyzed hydrothermal [5]. The novel microorganism is required to avoid pretreatment processes. The genetic engineering is used

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to find this solution for rich country. However in the developing country, utilization of local microorganisms have to be cultivated for bioethanol production.

Although commercial enzymes such as cellulase from *T. reesei* and β-glucosidase from *Aspergillus niger* were used for bioethanol production from cotton stalks [6]. However the pretreatment process is required before fermentation. Therefore this contributed to the high cost of bioethanol production.

A possible solution to lowering the production cost of bioethanol from agro-industrial lignocellulosic waste is to use low-cost enzymes that was produced from solid-state fermentation (S-SF) for example, a review of research studies in the production of cellulase using S-SF in basal mineral salt liquid medium (LM) using the abundance and inexpensiveness of cellulosic substrates and microorganisms, e.g. *T. reesei*, *A. niger* and *Penicillium* sp. and subsequent with submerged fermentation (SF) [7]. However the liquid medium in the research studied was used to adjust appropriate moisture content and for supplementary nutrients in S-SF which contained at 12 chemicals, e.g. Mary Mandels' mineral salts [8-10]. Therefore this liquid medium formula was increased cost and difficultly prepared.

Thus in this research, cassava residue, local of two strains microorganism (*T. reesei* and *S. cerevisiae*) and liquid medium new formula (consists of five substances) were performed at pH 5 and $24 \pm 2^{\circ}$C [11-13]. The product of S-SF, fresh fermented cassava residue with 55% w/w moisture content for 6 day incubation, was then dehydrated to obtain crude cellulase powder (12% w/w moisture content). The crude cellulase powder was collected in the seal plastic bag after pulverized. Submerged fermentation was performed using chopped dried pineapple peels and crude cellulase powder which were suspended in liquid medium for bioethanol fermentation.

II. Research Methodology

A. Biomass feedstock

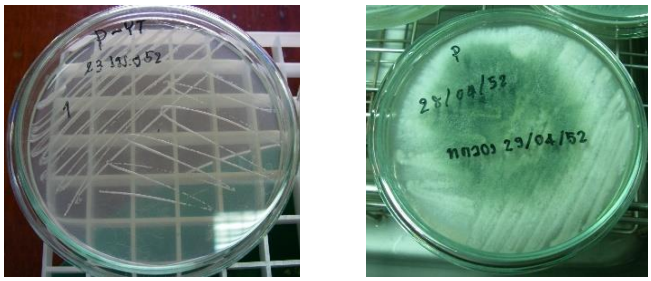
The cassava residue was obtained from General Starch Co., Ltd., a food processing company located in Thailand's northeastern province of Nakornratchasima, while Siam Winery Co. Ltd. contributed the fresh pineapple peels. The residues were sun-dried to reduce the moisture content to <13% w/w. The dehydrated substrates were subsequently hammer-milled to particle sizes of 0.5-1.0 mm prior to sterilization in an autoclave at 120°C for 20 min, after which the substrates were left to cool to room temperature (31°C).

B. Liquid medium

In this research, liquid medium (LM) was specially formulated and consists of 8 g urea ($(NH4)_2SO_4$), 15 g phosphate-potassium fertilizer (NPK-0-52-34), 1 g $CaHPO_4 \cdot 2H_2O$, 1 g $MgSO_4 \cdot 7H_2O$, 1 L reverse osmosis (RO) water, and the sugarcane concentration of which was 10, 20, 30 or 40 g/L. The preparation produced four sets of pre-S-SF LM according to sugar concentrations. The four sets of LM were pH-adjusted to pH 5 and then sterilized in the autoclave at 120°C for 20 min prior to leaving to cool to room temperature (31°C).

C. Preparation of co-cultured microorganisms

The co-culturing of *T. reesei* and *S. cerevisiae* was carried out by inoculating both strains onto potato dextrose agar (PDA) in the same Petri dish. The process began with streaking *S. cerevisiae* on PDA using an inoculating loop followed by transferred *T. reesei* from a PDA slant using an inoculating needle onto the center of the Petri dish, as shown in Fig. 1(a). The incubation period was 5 days at room temperature (31°C). Fig. 1(b) illustrates the co-cultured microorganisms after the 5-day incubation period.



(a) (b)

Figure 1 Co-cultured microorganisms on PDA dish: (a) initial incubation, (b) after five days of incubation

D. The optimal condition of fresh fermented cassava residue

The co-cultured microorganisms on the top surface of PDA plates were inoculated into the four sets of pH 5 LM individually before magnetically stirring for 30 min which were called inoculum starter of pH 5 LM. At the initially co-cultures in the inoculum starter of pH 5 LM was controlled of 10^7 cell/mL. Then, the optimal condition of fresh fermented cassava residue was carried out in 1 L glass bowls. The ratio of sterilized cassava residue (100 g) to inoculum starter of pH 5 LM were varied as 1:0.6, 1:0.8 and 1:1 w/v respectively. Each bowl was covered with wrap film perforated with an inoculating needle. Then cultivated them in incubation cabinet at $24 \pm 2^{\circ}$C for 7 days. The collection of samples and measurement of cell/mL were carried out daily in triplicate. Finally, once the optimal condition of fresh fermented cassava residue (i.e. 1:1 w/v for 6 days) was identified.

E. Crude cellulase powder production

Once fresh fermented cassava residue was performed at the optimal condition of 1:1 w/v for 6 days. And then production of fresh fermented cassava residue was carried out in a containers of 10 L Polypropylene (PP)

boxes for scaled up. Each PP box was filled with 625 g cassava residue, 125 g fresh fermented cassava residue and 750 mL pH 5 LM which had coconut sugar 30 g/L. Totally 6 PP boxes were investigated by cultivated in incubation cabinet at $24 \pm 2^{\circ}$C for 6 days. The obtained mixture of 6 boxes after 6 days incubated had an initial moisture content of 55 % w/w and 6,525 g fresh fermented cassava residue. These was further dried in a pilot scale oven until moisture content which was less than 12 % w/w. The cellulase powder was obtained after pulverization.

F. The application of crude cellulase powder to ethanol fermentation

The optimal condition for bioethanol in submerged fermentation was based on a previous study by Siwarasak P et al. [12]. The submerged fermentation was thus carried out in a 250 mL Erlenmeyer shaker flask filled with 8 g sterilized dried chopped pineapple peels and 6 g crude cellulase powder in 100 mL pH 5 LM containing 30 g/L coconut sugar, incubated for 5 days at room temperature (31°C). The collection of samples and measurements of ethanol, reducing sugar and cell concentrations and cellulase activity were undertaken in triplicate daily.

G. Analytical methods

The collections of samples of inoculum starter (1 g), fresh crude cellulase (1 g) and submerged fermentation supernatant (10 mL) for analysis were undertaken at the start of the experiment and every 24 h, while the powder sample (1 g) was collected only once after drying. Approximately 10 mL RO water was added to the solid samples individually prior to vortex mixing. The resultant suspensions were analyzed for cell concentration (cell/mL) using a haemacytometer (Boeco, Germany) with microscope which had a 40X magnification of the objective.

In addition, the cellulase activities of fresh crude cellulase, its powder and the supernatant were determined using filter paper [14]. After incubation at 50°Cfor 30 min, reducing sugar was determined with 3,5-dinitrosalicylic acid (DNS) reagent. The calibration curve for reducing sugar assay using glucose as a chemical standard [15]. Filter paper unit (FPU) is expressed in IU/gds of dry substrate. One unit (IU) of cellulase activity is defined as the amount of enzyme required to liberate one micromole of glucose per minute at 50°C.

Moreover, the supernatant was collected and tested for cell concentration, cellulase activity and reducing sugar, while the ethanol concentration was determined using the fresh distillation method [16]. All measurements were carried out in triplicate.

III. EXPERIMENT AND RESULTS

A. Effect of liquid medium volume and sugar concentration on starter inoculum

The cell concentration profile of co-cultured microorganisms for SSF with incubation time using 100 g cassava residue mixed with 60, 80 and 100mL of pH 5 LM individually containing 0.8, 1.6, 2.4 and 3.2 % w/w coconut sugar concentration are illustrated in Fig. 2-5, respectively, equivalent to a total of 12 conditions. The initially LM contains 10^7cell/mL co-cultured microorganisms and the incubation time is 7 days. Fig.6 compares the cell concentration under the 2.4 % and 3.2 % w/w coconut sugar concentration conditions. For 2.4 % w/w coconut sugar concentration and 100 mL of pH 5 LM, the maximum cell growth is achieved on day 6 of incubation as 3.2 % w/w at day 5; however, it has a lag phase of 3 days prior to the cell concentration increasing rapidly. By comparison, with 3.2 % w/w coconut sugar concentration, the lag phase is significantly reduced to 1 day.

This phenomenon is attributable to the higher reducing sugar concentration. Thus, the condition with 100 mL of pH 5 LM, 3.2 % w/w or 30 g/L coconut sugar concentration and 6 days of incubation is selected as the optimal condition for the preparation of fresh fermented cassava residue, which has the maximum cell concentration of 1.13×10^9 cell/mL. The higher cell concentration is implied cell growth of co-culture microorganisms. However the cell growth is inhibited by ethanol in submerged fermentation [7, 17]. Therefore, to induce the increasing cell concentration of co-culture microorganisms on day 2 of incubation, the coconut sugar concentration in inoculum starter of pH 5 LM is 30 g/L.

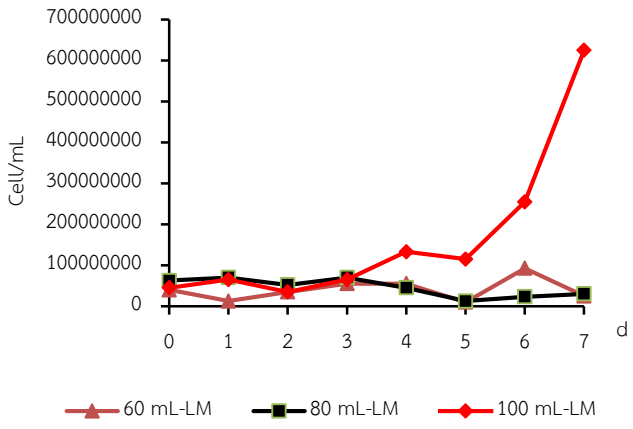


Figure 2 Cell concentration profile relative to cultivation time for S-SF under 100 g cassava residue mixed with 60, 80 and 100 mL inoculum starter of pH 5 LM containing 0.8 % w/w coconut sugar (SD = 2 %)

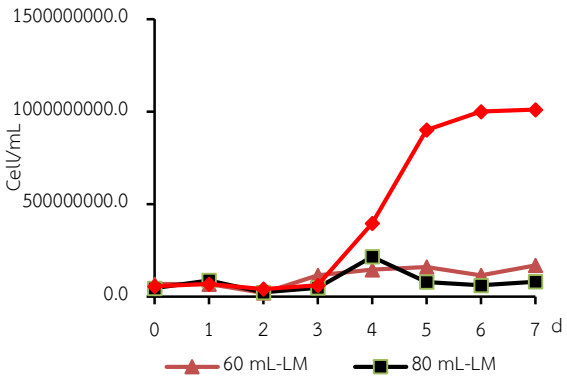


Figure 3 Cell concentration profile relative to cultivation time for S-SF under 100 g cassava residue mixed with 60, 80 and 100 mL inoculum starter of pH 5 LM containing 1.6% w/w coconut sugar (SD = 2)

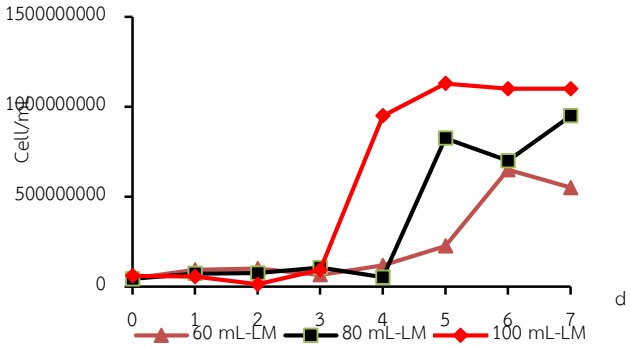


Figure 4 Cell concentration profile relative to cultivation time for S-SF under 100 g cassava residue mixed with 60, 80 and 100 mL inoculum starter of pH 5 LM containing 2.4% w/w coconut sugar (SD = 2%)

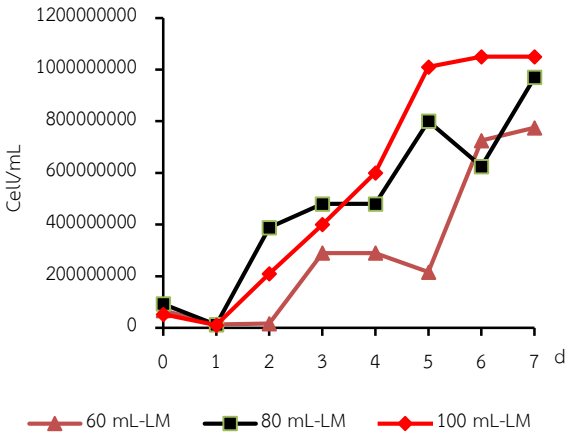


Figure 5 Cell concentration profile relative to cultivation time for S-SF under 100 g cassava residue mixed with 60, 80 and 100 mL inoculum starter of pH 5 LM containing 3.2%w/w coconut sugar (SD = 2%)

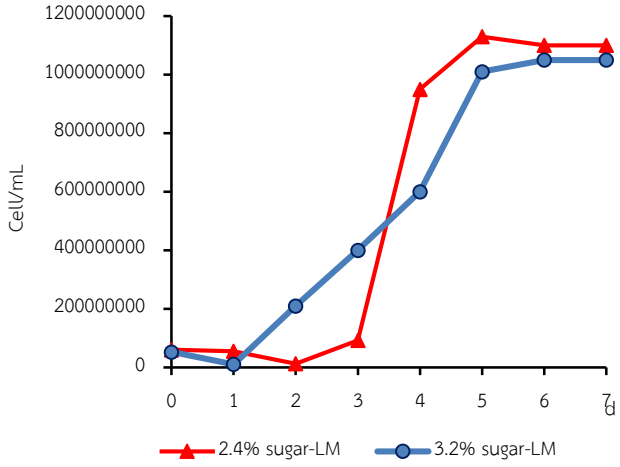


Figure 6 Cell concentration profile relative to cultivation time for S-SF under 100 g cassava residue mixed with 100 mL inoculum starter of pH 5 LM containing 2.4% and 3.2%w/w coconut sugar (SD = 2%)

B. *Cell growth and cellulase activity of fresh fermented cassava residue*

The larger-scale production of fresh fermented cassava residue was carried out in 10 L PP boxes filled with 625 g cassava residue, 125 g inoculum starter and 750 mL pH 5 LM with 30 g/L coconut sugar concentration prior to covering with perforated wrap film. The mixture had an initial moisture content of 55 % w/w and was incubated at $24 \pm 2^{\circ}$C for 6 days. In Fig.7, the maximum cell concentration of co-cultured microorganisms is 1.81×10^{9} cell/mL on day 6 of incubation. It was found that the moisture content of 55 % w/w is suitable to produce fresh fermented cassava residue. If the moisture content higher than 55 % w/w (data was not show) could contribute to low substrate porosity and reduce oxygen penetration. Similarity, if the moisture content lower than 55 % w/w cell concentration is also reduced due to poor accessibility to nutrients. [18, 19]. The effect of low moisture content in S-SF on reduction in nutrients solubility, low degree of swelling and high water tension had been reported [20].

And also, Fig. 7 illustrates initially fresh fermented cassava residue had cellulase activity of 3.35 IU/gds due to fresh fermented cassava residue of 125 g was used. However the cellulase activity is 8.64 IU/gds on day 5 of incubation. The decline in activity during days 7 is possibly attributable to the reduction of substrate and nutrients [21]. Interestingly, utilization of 30 g/L coconut sugar in pH 5 LM, mycelia and spores could be higher than the others. Therefore this could be shortens of 1 day lag phase [21, 22]. The cellulsae activity of crude cellulase powder increasing from 8.6 to 27.7 IU/gds was found, as shown in Fig. 8. In Fig. 9 was shown pictures of the fresh fermented cassava residue and crude cellulase powder of this research

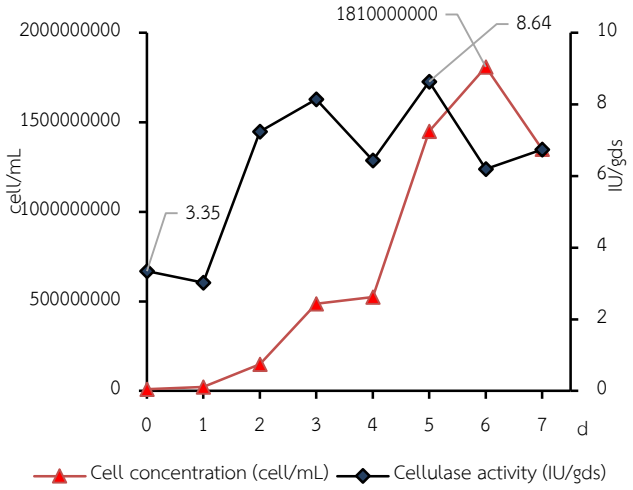


Figure 7 Cell concentration profile and cellulase activity relative to incubation time for S-SF under 625g cassava residue mixed with 125g fresh fermented cassava residue and 750mL of pH 5 LM containing 30g/L coconut sugar (SD = 3%)

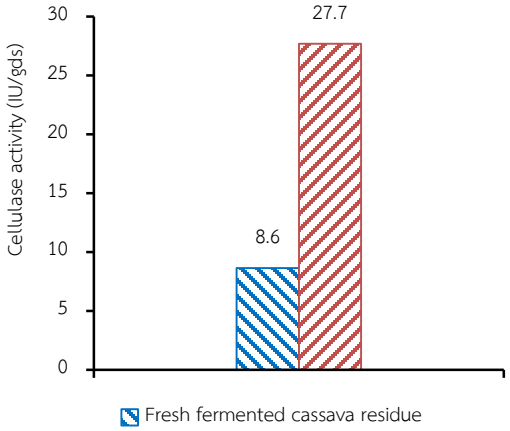


Figure 8 Cellulase activity of fresh fermented cassava residue (55 % w/w moisture content) and crude cellulase powder (12 % w/w moisture content) (SD = 3%)

(a)

(b)

Figure 9 The product from S-SF: (a) fresh fermented cassava residue (before drying) and (b) crude cellulase powder (after drying and pulverization)

C. Crude cellulase powder for ethanol fermentation

The batch production of ethanol was undertaken using submerged fermentation in a 250 mL Erlenmeyer shaker flask filled with 8 g chopped dried pineapple peel, 6 g crude cellulase powder, and 100 mL of pH 5 LM which contained 30 g/L coconut sugar concentration at room temperature 31°C. The flask was continuously shaken at 100 rpm for 5 days. The initial ethanol concentration was 7.7 g/L, due to this was product in cellulase powder from S-SF. However the maximum ethanol was achieved of 42.1 g/L at day 4 incubation time as shown in Fig. 10. Because of there was initially cellulase activity in cellulase powder of 27.7 IU/gds. Therefore cell concentration increasing rapidly corresponding to reduction of reducing sugar and the maximum ethanol concentration was obtained, as illustrated in Fig. 10 and 11.

The co-cultured microorganism of *T. reesei* and *S. cerevisiae* simultaneously hydrolyze cellulose of chopped dried pineapple peel into eyhanol due to cellulase activity is active from the initial. The reduction of cellulase activity was found less active in subsequent day because of the supernatant sample was collected and analyzed. This is limitation of the study that could not analyze cellulase activity from solid fermented. Therefore chopped dried pineapple peel was fermented directly without pretreatment to remove lignin with cellulase powder via submerged fermentation. As such, the powder is convenient for use, particularly for ethanol fermentation, in addition to the inexpensiveness of the microorganisms and agro-industrial residues.

This research study was found that crude cellulase powder can be produced from local microorganisms. Cellulase activity is higher than previous research as shown in Table 1. One reason is two strains of *T. reesei* and *S. cerevisiae* which can be cultivated in the same substrate. Then these can be hydrolyzed cellulose and simultaneously converted to ethanol. Even though incubation time for crude cellulase powder in this study is 144 hours. However the level of this fermentation is higher than the previous study. In addition the abundance of cassava residue and pineapple peel waste were used in Thailand.

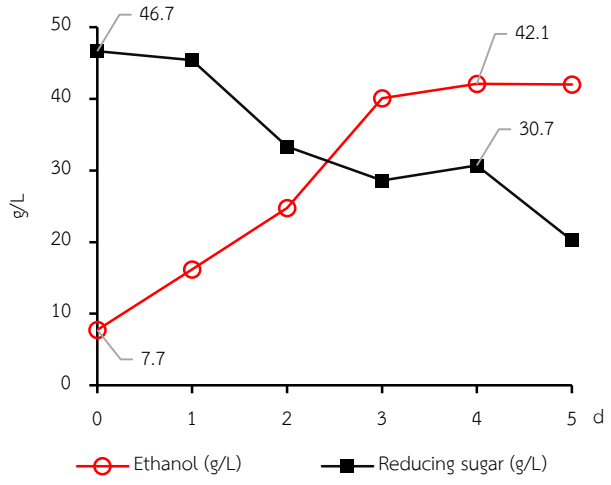


Figure 10 Profile of concentrations of reducing sugar and ethanol for submerged fermentation using dried chopped pineapple peels and crude cellulase powder in pH 5 LM (SD = 3%)

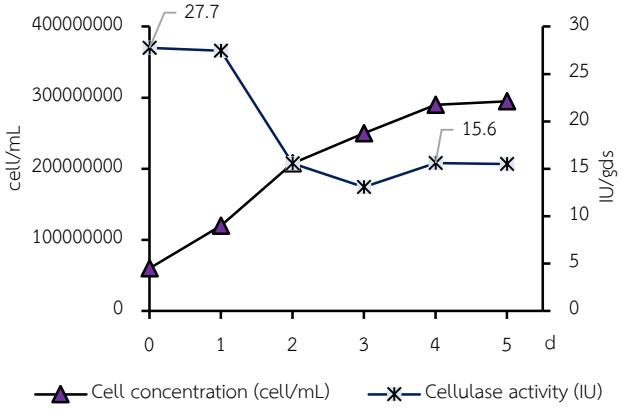


Figure 11 Profile of cell concentrations and cellulase activity for submerged fermentation using dried chopped pineapple peels and crude cellulase powder in pH 5 LM (SD = 3%)

IV. Conclusion

This research is concerned with the production of fresh fermented cassava residue by solid state fermentation (SSF) using the co-cultured microorganisms of *S. cerevisiae* and *T. reesie*. The microorganisms were co-cultured at room temperature 31°C for 7-12 days. And then inoculated on cassava residue and was incubated at $24 \pm 2^{\circ}$C for 5 days. The resultant fresh fermented cassava residue (55%w/w moisture content) was then transformed into crude cellulase power (12%w/w) through the drying and grinding processes.

The optimal preparation condition of inoculum starter in 1 L glass bowl was 100 g cassava residue mixed with 100 mL pH 5 LM of 30 g/L coconut sugar concentration incubated at 24±2°C for 6 days. The production of fresh fermented cassava residue was subsequently enlarged in scale and carried out in 10 L PP boxes filled with 625 g cassava residue, 125 g inoculum fresh fermented cassava residue starter and 750 mL pH 5 LM of 30g/L coconut sugar concentration prior to covering with perforated wrap film, incubated at 24±2°C for 6 days. The cell concentration and cellulase activity of the resultant fresh crude cellulase are 1.81×10^{9} cell/mL and 8.64 IU/gds, respectively.

The crude cellulase powder production is performed by drying and grinding fermented cassava residue after 6 days S-SF. Due to its ease of use in bioethanol production [13], crude cellulase powder is therefore a promising enzyme for ethanol production from agro-industrial wastes. However, to address the small-scale limitation, further optimization of the fermentation condition to suit the larger-scale ethanol production is advisable.

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