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Evaluation and comparison of microbial cells disruption methods for extraction of pyruvate decarboxylase

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Abstract

An enzymatic biotransformation process based on extracted pyruvate decarboxylase (PDC) overcomes the problem of by-product benzyl alcohol production. Seven methods of cells disruption which included application of glass beads, freezing/thawing sequence, silver nanoparticles, ultrasonication, freezing/thawing sequence with glass beads, freezing/thawing sequence with silver nanoparticles, and freezing/thawing sequence with ultrasonication were investigated to choose the best method for partial isolation of PDC from *Candida tropicalis* TISTR 5350. Ultrasonication was an effective method for cells disruption with the corresponding specific PDC activity of 0.36 ± 0.01 U/mg of protein. Ultrasonication method should thus be selected as the method of choice to preserve enzyme activity. The PDC from *C. tropicalis* TISTR 5350 released from ultrasonic cells disruption method was prepared for partial purification in comparison with 40-60% (w/v) ammonium sulphate and 50% (v/v) acetone precipitation techniques. Specific PDC activity, purification and percentage recovery (yield) of precipitated PDC enzyme based on 50% (v/v) acetone at 4ºC were higher (0.53 ± 0.02 U/mg protein, 1.24 ± 0.10, and 94.41 ± 2.12 %, respectively) than the precipitation obtained using the 40 to 60% (w/v) ammonium sulphate saturation (0.49 ± 0.01 U/mg protein, 1.13 ± 0.07, and 92.87 ± 2.50%, respectively). The result indicated that the precipitation of PDC using the 50% (v/v) acetone was the most effective strategy for partially purifying PDC and suitable for application in a commercial process which had a relatively low cost and simple process.

Introduction

Microorganisms consisted of a semipermeable, tough, rigid, outer cells wall surrounding the cytoplasmic membrane, and cytoplasm. The cytoplasm was made up of nucleic acids, proteins, carbohydrates, lipids, enzymes, inorganic ions, vitamins, pigments, inclusion bodies, and about 80% water. In order to isolate and extract these substances from inside the cells, it was necessary to break the cells wall and protoplasmic membrane (APV, 2009). Cells could be disrupted in various ways, which included; (1) mechanical methods such as homogenization (shear force), cells rupture by pressure, sonication, grinding, osmotic shock, as well as freezing and thawing sequence; (2) enzymatic method such as lysozyme, zymolase, cellulase, glycanase, protease, and mannase; (3) chemical methods such as treatment with detergents, silver nanoparticles, and solvents (Stephanova and Topouzova, 2001).

An enzymatic process with free pyruvate decarboxylase (PDC) successfully eliminated benzyl alcohol formation since there were no electron regeneration donors (e.g. NADH) (Shin and Rogers, 1996; Rosche *et al*., 2002). PDC extracted from the yeast has been intensively studied in the development of enzymatic PAC production processes which was a precursor for the commercial production of ephedrine and pseudoephedrine. These substances were used primarily as bronchial dilators and nasal decongestants (Suresh *et al*., 2009). Moreover, PAC production should preferably exhibit high levels of PDC (Chen, 2006).

Candida tropicalis could utilize wide variety of carbon sources including many saccharides, phenols, alkanes, alkane derivatives, and fatty acids (Kawachi *et al*., 1997). This yeast was considered strong candidate for thermotolerance and ethanol tolerance required to produce ethanol from lignocellulosic biomass (Jamai *et al*., 2001). As previous studied from Tangtua *et al.* (2013), *C. tropicalis* TISTR 5350 was the most interesting candidate microbial strain which observed the highest PDC activities (0.39 ± 0.06 U/ml) and PAC production (19.83 ± 3.36 mM). The cytoplasm of yeast cells was a rich source of bio-products (proteins, cytoplasmic enzymes, polysaccharides, etc.). *C. tropicalis* was a simple eukaryotic cells with a relatively rigid cells wall in addition to cell membrane. Hence, efficient breakage of the cells walls was a necessary step to recover

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intracellular compounds (Liu *et al*., 2013).

The main objective of this study was to consideration of the best method for partial isolation of intracellular PDC enzyme. *C. tropicalis* TISTR 5350 was used to compare seven methods of cells disruption. The PDC enzyme released from the most appropriate cells disruption method was prepared for partially purification in comparison with ammonium sulphate and acetone precipitation techniques.

Material and Methods

Cultivation of microorganism and sample preparation

Candida tropicalis TISTR 5350 were ordered from Thailand Institute of Scientific and Technological Research (TISTR). The frozen stock of 1.0 ml was thawed and cultivated in a 10 ml inoculation medium for later propagation in a 100 ml scale. The cultivation was carried out at 30°C on a rotary shaker at 200 rpm for 24 h (Laluce *et al*., 2009). Cells pellet was recovered from 10 ml culture broth and washed twice with 10 ml distilled water. This was performed prior to resuspension in citrate buffer (pH 6.0).

Cells disruption methods

Each method was divided in to two groups which included freezing and thawing cycles prior to cells disruption and without freezing and thawing cycles.

Freezing and thawing methods

The suspension of wet cells pellet in 200 mM citrate buffer (pH 6.0 / 4.5 M KOH) was immersed in liquid nitrogen and then placed in a 30°C water bath for three cycles of freezing and thawing. Non-freezing and thawing cycles were used as control.

Glass bead-vortexing methods

The suspension of wet cells pellet was added with glass beads (0.55 mm diameter) at volume ratio of 1:1. The mixture was vortexed at a maximum speed for 1 min with the similar cooling time in an ice/water mixture for three cycles.

Silver nanoparticles methods

Silver nanoparticles were obtained from the courtesy of Sensor Research Unit, Department of Chemistry, Faculty of Science, Chulalongkorn University. The concentration of silver colloidal suspension was 0.1% (w/v) which was stabilized in 2% (w/v) soluble starch. The average size and size distribution were 6.03 ± 2.60 and 5 - 20 nm, respectively. Active cultures of *C. tropicalis* TISTR 5350 cells were treated with 15 µg/ml silver nanoparticles solution for 3 min. The mixture was vortexed at a maximum speed (1,300 x *g*) for 1 min and cooled down for 1 min in ice / water mixture.

Ultrasonication methods

Cells were disrupted by ultrasonication with the capacity to operate at a maximum power output of 1.2 kW and a frequency of 20 kHz. The cells were sonicated using 10 ml aliquots in 50 ml glass jar with screw cap for 5 s at amplitudes from 1% to 60%. The total sonication time was 6 min. The ultrasonication treatments were completed in an ice bath to minimize losses of enzyme activity as a result of the temperature increase.

After each treatment strategy, cells debris was removed by centrifugation at 2,822 × *g* for 15 min and the supernatant was collected and stored at -20°C for enzyme assays and protein concentration estimation.

Comparison of acetone and ammonium sulphate precipitation

The most appropriate method for cells disruption was prepared for enzyme purification. Supernatant from centrifugation method was collected for subsequent investigation suitable of PDC partial purification method.

Acetone precipitation

Acetone precipitation of the crude extract in the concentration level of 50% (v/v) at 4ºC was employed to obtain partially purified PDC (Leksawasdi, 2004; Sandford *et al.*, 2005). Precooled acetone at -20°C was slowly added to the crude PDC extract until the acetone concentration of 50% (v/v) was reached. After the mixture was stood overnight at 4°C, the precipitate was removed by centrifugation at 12,100 × *g* for 15 min at 4°C. The precipitate was dissolved in 0.2 M citrate buffer (pH 6.0/KOH) for subsequent enzyme activity and protein concentration analysis.

Ammonium sulphate precipitation

Ammonium sulphate was transferred to the crude PDC extract until the concentration of 60% (w/v) saturation was reached. After the mixture was stood overnight at 4°C (Fatima and Husain, 2007), the precipitate was removed by centrifugation at 12,100 × *g* for 15 min at 4°C. The precipitate was dissolved in 0.2 M citrate buffer (pH 6.0/KOH). The solution was dialyzed against 10 times diluted citrate buffer at 4°C for 24 h using a dialysis bag (Sarethy *et al*., 2012). The solution was collected for subsequent

enzyme activity and protein concentration analysis.

Determination of enzyme activity and protein concentration

PDC carboligase activity was measured as a formation of PAC in 20 min at 25°C from 80 mM benzaldehyde and 200 mM pyruvate in carboligase buffer. One unit (U) carboligase activity was defined as the amount of enzyme that produced 1 µmol PAC from pyruvate and benzaldehyde per min at pH 6.4 and 25°C in a carboligase assay as specified by Rosche *et al.* (2002). The detection of protein concentration was performed according to Bradford (Bradford, 1976) using bovine serum albumin. Specific carboligase activity was determined based on protein concentrations in the sample and expressed in units of enzyme per milligram protein (U/mg). The degree of purification or purification factor (n-fold purification) could be obtained from the following relationship (Irshad *et al.*, 2012):

Purification factor = specific enzyme activity after purification step / initial specific enzyme activity before purification

Results and Discussion

Comparison of cells disruption methods

In order to monitor cells disruption, the total protein (mg/ml) and activities of PDC was assayed in the extract. The highest specific PDC activity of 0.37 ± 0.04 U/mg protein was obtained from the freeze-thaw cycles prior to ultrasonication method with the corresponding protein concentration of 3.00 ± 0.28 mg/ml as indicated in Table 1. This was not significantly different ($p > 0.05$) from ultrasonication without freeze-thaw cycles with the corresponding PDC activity of 0.36 ± 0.01 U/mg protein.

Ultrasonication was one of the most commonly used mechanical cells disruption methods for extraction of intracellular products. This method created high shear force by high frequency ultrasound (above 16 kHz) (Chemat *et al*., 2011). Mechanism of cells disruption by ultrasound was normally associated with the cavitation phenomena (Vargas *et al*., 2004). Cavitation was the combination of formation, growth, and collapse of gas and vapor bubbles, which were induced by the action of intense sound waves (Gogate, 2011). In the collapse phase of cavitation bubbles, a large quantity of sonic energy was converted to mechanical energy in the form of intense elastic shock waves in which the pressure reached as high as thousands of atmosphere near the point of collapse (Borthwick *et al*., 2005). As a consequence, the use of ultrasound leads to a better cells disruption and faster mass transfer between the solvent and host material.

Table 1. Comparison of cells disruption methods

Cell disruption methods	Volumetric PDC activity (U/ml)		Specific PDC activity (U/mg protein)		Protein concentration (mg/ml)	
Whole cells (control)	0.00 ± 0.00	E	0.00 ± 0.00	D	0.00 ± 0.00	D
Freezing and thawing	0.20 ± 0.01	D	0.21 ± 0.01	C	0.72 ± 0.04	D
Glass bead	0.70 ± 0.01	BC	0.26 ± 0.01	B	0.94 ± 0.07	A B
Silver nanoparticles	0.47 ± 0.01	C	0.22 ± 0.01	C	2.74 ± 0.15	B
Ultrasonication	0.86 ± 0.03	B	0.36 ± 0.01	A	2.16 ± 0.12	B
Freezing and thawing + Glass bead	0.90 ± 0.02	B	0.36 ± 0.03	A	2.57 ± 0.13	B
Freezing and thawing + Silver nanoparticles	0.45 ± 0.03	C	0.25 ± 0.03	B	1.86 ± 0.07	C
Freezing and thawing + Ultrasonication	1.05 ± 0.05	A	0.37 ± 0.04	A	3.00 ± 0.28	A

The number with the same alphabet (A - E) indicated no significant difference ($p > 0.05$) for comparison between different rows of the same columns.

The methods by freezing and thawing sequence prior to bead-vortexing resulted in a relatively high specific PDC activity of 0.36 ± 0.03 U/mg protein. However, the PDC activity from this method was not different statistically ($p > 0.05$) from ultrasonication method. Brooks (2013) stated that glass bead method was the process involving mechanically disrupting cells by shearing forces caused by the movement of the liquid in which the cells were suspended and ground with glass beads on a vortex mixer. Mayerhoff *et al.* (2008) evaluated *C. mogii* cells disruption by comparing processes employing glass beads and ultrasound in conditions. Disruption with glass beads showed to be the most efficient method for xylose reductase release and the highest specific activity of 0.13 U/mg of protein.

Specific PDC activity from whole cells (untreated) was not detected which was found to be significantly different ($p \leq 0.05$) from those that underwent freezing and thawing method and 15 µg/ml silver nanoparticles with the corresponding specific PDC activity of 0.21 ± 0.01 and 0.22 ± 0.01 U/mg protein, respectively. These results indicated that the use of freezing/thawing cycle enhanced cells disruption efficiency. According to Burns *et al.* (1998) who stated that freezing/thawing cycle was an efficient and relatively gentle process of cells disruption. The holes at cells envelope could be formed with minimal impacts on the overall integrity of cells. Moreover, Negron (2010) also proved that the technique involved freezing a cells suspension in dry ice or freezer and then thawing the material at room temperature or 37°C. This method of lysis caused cells to swell and ultimately broke due to forming of ice crystals during the freezing process and then contracted during thawing. Multiple cycles were necessary for efficient lysis, and the process could be quite lengthy.

Silver nanoparticles were closely associated with the formation of pits in the cells wall. The accumulation of silver nanoparticles in the microbial membrane caused the permeability, releasing desired biomolecules from within the cells (Sondi and Salopek-Sondi, 2004). However, silver nanoparticles

Table 2. Partial purification of PDC from *C. tropicalis* TISTR 5350 using 50% (v/v) acetone and 40-60% (w/v) ammonium sulphate concentrations

Purification steps	Total volumetric activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)	Purification (folds)	Recovery (%)
Crude	0.68 ± 0.04 A	1.56 ± 0.02 A	0.44 ± 0.02 C	1.00	100.00
50% (v/v) Acetone	0.63 ± 0.01 A	1.19 ± 0.02 BC	0.53 ± 0.02 A	1.24 ± 0.10 A	94.41 ± 2.12 A
40 - 60% (w/v) Ammonium sulphate	0.62 ± 0.02 A	1.28 ± 0.02 B	0.49 ± 0.01 B	1.13 ± 0.07 B	92.87 ± 2.50 A
Supernatant	0.29 ± 0.02 B	0.70 ± 0.03 C	0.42 ± 0.03 C	0.98 ± 0.10 C	43.41 ± 4.50 B

The number with the same alphabet (A - C) indicated no significant different ($p > 0.05$) for comparison between different rows of the same columns.

method was significantly lower ($p \leq 0.05$) than glass bead and ultrasonic method. It was possible that the membrane of microorganism contained of lot of sulphate-containing enzymes and it had been proposed that silver ions interacted with these sulphate-groups and thereby changed the membrane morphology by inactivating enzymes. This inactivation made the membrane vulnerable and easier to penetrate for silver ions. Inside the cells, silver ions continue to destroy different parts of the cells by interacting with sulphate-groups, which were often located in the active site of enzymes. This interaction with the active site caused inactivation of the enzymes (Kildeby *et al*., 2005).

The study of the release of PDC from *C. tropicalis* TISTR 5350 cells disruption by freeze-thaw cycles prior to ultrasonication method had although shown the highest specific enzyme activity. This was not significantly different ($p > 0.05$) from ultrasonication without freeze and thaw cycles process. Hence, ultrasonic without freeze and thaw cycle was adopted as the preferred method for producing cells free extracts of PDC activity in *C. tropicalis* TISTR 5350 which was not a much more time consuming process (20 min).

Comparison of PDC partial purification method

From Table 2, fractions precipitated with the acetone concentration level of 50% (v/v) exhibited the highest specific PDC activities, purification fold, and recovery percentage of 0.53 ± 0.02 U/mg protein, 1.24 ± 0.10, and 94.41 ± 2.12, respectively. This was compared to the 40 - 60% (w/v) ammonium sulphate in which the corresponding specific PDC activity (0.49 ± 0.01 U/mg protein), purification fold (1.13 ± 0.07) and enzyme recovery (92.87 ± 2.50 %) were attained. Such result was similar to Mahdavi *et al.* (2010) who precipitated α-amylase from *Bacillus cereus*. The fraction obtained at 50% (v/v) acetone showed the highest specific enzymatic activity of 50 U/mg protein.

The miscible acetone solvents by addition of acetone to the solution would decrease the dielectric constant of water. This effect allowed proteins to come close together. The solvation layer around the protein decreased as the organic solvent progressively displaced water from the protein surface. The proteins could aggregate by attractive electrostatic and dipole forces due to smaller hydration layers. Moreover, important parameters for protein precipitation were temperature, pH, and concentration of protein in the solution. In addition, the efficiency of this method depended on the protein molecules attaching to the other proteins in solution to form aggregates (Israelachvili, 2011).

The specific enzyme activity, purification fold, and recovery percentage from acetone precipitation were not only higher than ammonium sulphate precipitation, but this method also had the advantage of being simple and convenient which was appropriated for commercially process. This was compared to the ammonium sulphate procedure which had numerous steps, cumbersome, and time consuming. The dialysis bag must be used to remove the salt which was relatively expensive and unsuitable for use in routine process. In term of commercial PDC purification, the precipitation of proteins by 50% (v/v) acetone was accounted for the cost of 1.05 Baht/sample (0.21 Baht/ml). The economical total enzyme activity from acetone method (1.08 U/Baht/ml) was higher than 40 - 60% (w/v) ammonium sulphate precipitation method (0.005 U/Baht/ml) with the corresponding cost of 0.12 Baht/sample (0.18 Baht/g). The additional cost relevant to ammonium sulphate method involved the dialysis step to remove the salt from the precipitate with the relatively high price of dialysis tube at 1,460 Baht/meter. In addition, the salting out method was a much more time consuming process (48 h, in comparison to 28 h of acetone method). A technique of ammonium sulphate precipitation was generally used in a small scale (lower than 500 ml) (Cutler, 2004; Yadav, 2005) and was considered unsuitable for a very large scale (up to 500 ml) (Yadav, 2005) preparation. These results suggested that cold acetone was probably the most effective agent for PDC purification.

Conclusion

Ultrasonication method was the most effective cells disruption method which revealed the specific activity of 0.36 ± 0.01 U/mg protein. Moreover, sonication did not require the addition of beads or chemicals such as NaCl (for osmotic shock), which might need to be removed later in the process, thus increasing processing cost. Ultrasonic devices could be scaled-up and operated continuously.

The PDC enzyme released from cells was further

prepared for partial purification in comparison with ammonium sulphate and acetone precipitation techniques. The highest specific enzyme activity (0.53 ± 0.02 U/mg protein), purification factor (1.24 ± 0.10), and activity recovery (94.41 ± 2.12 %) were obtained from the precipitated enzyme by 50% (v/v) acetone. In addition, the precipitation of PDC using the 50% (v/v) cold acetone was suitable for application in a commercial process which had a relatively low cost and simple process.

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