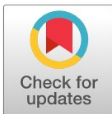

International Journal of Applied and Physical Sciences
volume 4 issue 3 pp. 110-115 doi: https://dx.doi.org/10.20469/ijaps.4.50005-3

Efficient Precipitation Methods of Inulinase from Endophytic Bacteria *Bacillus aquimaris* Isolated from Jerusalem Artichoke

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Abstract: Inulinase is an enzyme that hydrolyzes inulin to oligosaccharides used in the food industry. Inulin is a source of fructose production using inulinase hydrolysis in one step. Jerusalem artichoke (*Helianthus tuberosus L.*) is a high inulin plant that was accumulating inulin in tubers. Thus, the endophytic bacterium, *Bacillus aquimaris* was isolated from Jerusalem artichoke to determined inulinase activity. This bacterium produced high inulinase when grown in Luria Bertani medium containing 1% inulin as the carbon and energy source with incubation temperature at 37°C for 20 hours and shaking speed of 150 rpm. The crude enzyme showed specific inulinase activities at 1.61 U/mg protein after incubation at 55°C for 20 min in the presence of the inulin substrate. The suitable method of enzyme concentration was studied in this work for purification and characterization in further. There were four methods for protein precipitation using ammonium sulfate, ethanol, butanol, and 2-steps of salt and alcohol. The best-precipitated protein method was 50% of ethanol, giving 53% protein recovery with specific activity at 26.21 U/mg. This method was efficient with inulinase not only protein concentration but also protein purification (16.24 fold).

Keywords: *Inulin, inulinase, endophytic bacteria*

Received: 17 July 2018; ***Accepted:*** 18 September 2018; ***Published:*** 14 November 2018

I. INTRODUCTION

Inulin is a polysaccharide (fructan type) of linear chain β-(2,1) linked fructan residues attached to a terminal sucrose molecule [1]. Inulin accumulates in several plants such as leak, onion, garlic, tulip, chicory and Jerusalem artichoke. The beneficial effects of inulin and oligofructose have been reported in a large number of health promoting functions; thus, there are wide applications in various types of foods like confectionery, fruit preparations, milk desserts, yogurt and fresh cheese, baked goods, chocolate, ice cream and sauces. Inulin can also be used for the preparation of fructose syrups [2] by hydrolysis of enzymes known as inulinases. Inulinase (β-fructosidase, EC 3.2.1.7) catalyze the β-(2,1) link of inulin. The characteristic of inulinase has two forms; (i) exo-inulinase degrade β-(2,1) linked bond of non-reducing area, producing fructose [3]; (ii) endo-inulinase degrade a random area of substrate, giving inulo-oligosaccharide, which is low in sweetener, prebiotic and used as component of a healthy diet [4, 5].

Fructose is a basic monosaccharide sugar that is high sweetness. It can use as a sweetener which is a good

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choice for those affected by obesity and diabetes mellitus. Moreover, it is widely used in many foods, beverages and the pharmaceutical industry [6]. Fructose can be used as a raw material in fermentation industries such as the production of ethanol, acetone and butanol [7]. These industries normally use chemical reactions to change sucrose into fructose including fructose syrup. Mostly, sucrose is produced from starch using many steps of enzymes, such as -amylase, amyloglucosidase and glucose isomerase. To use a single-step enzymatic reaction for fructose production, inulinase is a proper enzyme to give yields up to 95% from inulin substrate [2].

Endophytic bacteria that live inside high inulin plant as Jerusalem artichoke should have inulinase activity specific to inulin. Also, Jerusalem artichoke demonstrates good growth in poor soil, high tolerance to various plant diseases and very high carbohydrate yields [8]. This plant is 11-20% by weight of carbohydrates, 70-90% of these being inulin [9]. Therefore, the endophytic bacterium, *Bacillus aquimaris* isolated from Jerusalem artichoke, which showed high inulinase activity in medium containing inulin was used in this study for the determination of a suitable procedure of protein precipitation. This method will be considered for the purification and characterization of the enzyme.

II. LITERATURE REVIEW

Glycoside Hydrolases (GH) play important roles in various biological processes in plant, fungi, and bacteria as well as the biosynthesis of glycans including plant defenses, cell wall metabolism, signaling, and the mobilization of storage reserves. The GH enzymes show a common structure of β-propeller catalytic domain with three conserved amino acids including aspartate (D) in 'WMN**D**PNG' motif, aspartate (D) in R**D**P motif and glutamate (E) in '**E**C' motif. This active site located in the deep axial pocket of the catalytic domain. The propeller structure has 5 of fold blades that each blade exhibits four antiparallel β-strands with the classical 'W' topological structure around the center of the axis in N-terminal domain. It enclosed a negatively charged cavity of the active site. All of inulinase enzymes contain the consensus-conserved motifs R-D-P-K-V-F-W-H and W-M-N-D-P-N-G. The consensus motif (WMNDPNGL) acts as a nucleophile, and another consensus-conserved motif (RDPKVF) has a key role in catalytic activity [10]. Other conserved motif 'SVEVF', 'RDP', 'EC (V) P', 'Q' and 'FS (T)' involved in catalytic of exo-inulinase, except on motif 'SVEVF' which did not found in exo-inulinase producing yeast; therefore, yeast do not produced any endo-inulinase [11]. In exo-inulinase and endo-inulinase are conserved in motif 'SVEVE' that involved to attack inulin and levan substrates [12].

There are many methods for protein precipitation; the most popular one is ammonium sulfate precipitation as it has much a higher solubility than any of the phosphate salts. The protein solubility usually decreases at higher salt concentrations, leading to protein concentration; this effect is called salting-out. Organic solvents can also precipitate proteins, such as ethanol and butanol. The organic solvents decrease the solubility of proteins at low temperatures; however, alcohols and other organic solvents are protein destabilizers and can denature proteins at high concentrations or high temperatures due to their favorable interactions with hydrophobic groups [13]. For tertiary butanol, it is normally formed miscible with water, but upon the addition of enough salt. Therefore, t-butanol is used with ammonium sulfate by separating protein in interphase between the lower aqueous and upper t-butanol phase, termed three-phase partitioning [14, 15].

There had many reports for inulinase precipitation from bacteria. [16] used 60% of ethanol to precipitated inulinase of *Arthrobacter aurescens* SK8.001 with a 1.75-fold increase in the specific activity. The chilled ethanol at 85% showed the ability of inulinase precipitation with 80% recovery in *Kluyveromyces marxianus* YS-1 [4]. For salting out method, the ammonium sulfate concentration was a different application in range of 20-100%, e.g. inulinase of *Bacillus cereus* MU-31 was precipitated at 80% [17], inulinase of *Pseudomonas putida* was 40-80% [18] and inulinase of *Xanthomonas campestris* pv. *phaseoli* KM24 was 20-100% [19]. The 3 phase protein portioning of inulinase was reported in *Aspergillus niger* using 30% of ammonium sulfate with 1.0 : 0.5 v/v ratio of t-butanol, giving 88% recovery and 10.2 fold of purification [20]. It is likely that the inulinase enzyme can precipitate in both salts and alcohol depending on suitable condition in each method.

III. MATERIALS AND METHOD

A. Bacterial Cultivation

The endophytic bacteria, *Bacillus aquimaris* 3.13 isolated from Jerusalem artichoke was transferred to LB broth containing 1% inulin, grown at 37°C for 20 h with shaking (150 rpm). The cell was precipitated at 10,000 rpm of centrifugation for 10 min. The supernatant was collected for the crude enzyme.

B. Determination of Inulinase Activity

The crude enzyme (0.5 ml) was added into 1.5 ml of 50 mM sodium phosphate buffer (pH 6.0) containing 1% inulin. The mixture was incubated at 55°C for 20 min;

the reaction was stopped on ice for 5 min. Inulinase activity was assayed by the determination of reducing sugars using DNS method [21]. Definition of inulinase (1U) is defined as one micromole of reducing sugar produced by the amount of enzyme in a minute under the assay condition. Protein contents were measured by Lowry's method [22], using bovine serum albumin as a standard.

C. Test of a Suitable Method for Inulinase Concentration

1) Ammonium sulfate precipitation: The 10 ml of crude enzyme was mixed with ammonium sulfate at 20, 40, 60 or 80% and then continuously stirred during incubation at 4°C for 1 h. The pellets were collected by centrifugation at 8,000 rpm for 15 min. The pellets were dissolved in a small amount of sodium phosphate buffer (50 mM, pH 6.0). The protein contents and inulinase activity were determined to find an optimum concentration of salts.

2) Alcohol precipitation: The crude enzyme (10 ml) was mixed with ethanol or butanol in the ratio of 20, 30, 40 or 50%, was stirred at 4°C for 1 h. The pellets were kept at 8,000 rpm for 15 min by centrifugation and then dissolved with sodium phosphate buffer (50 mM, pH 6.0). The protein and enzyme activity was determined to find a suitable and optimum concentration of alcohol.

3) 2-Steps of salt and alcohol: This method was adapted from the three-phase partitioning (TPP) [6]. The 10 ml of crude enzyme were mixed with ammonium sulfate (30% w/v), after that 1:0.5 ratio of t-butanol was added. The mixture was incubated at 25°C for 1 h; the centrifugation at 2,000 rpm was performed for 15 min. The protein was dissolved with sodium phosphate buffer (50 mM, pH 6.0). The protein and enzyme activity was determined.

IV. RESULTS AND DISCUSSION

There were four methods for precipitation of inulinase from *B. aquimaris*: ammonium sulfate, ethanol, t-butanol, and 2-steps of salt and alcohol. The precipitation of inulinase enzyme by ammonium sulfate was varied from 20-80% saturation (Figure 1(a)). The highest protein precipitation was achieved at 20% ammonium sulfate saturation, giving total enzyme activity at 138 U and high amounts of protein. It was similar to some enzymes that precipitated at low concentration of salt; for example, the 30-40% of ammonium sulfate was used to precipitate xylanases produced from *Thermomyces lanuginosus* and its mutant by overnight saturation at 0°C [23]. *Gymnoascella citrina* produced novel endoglucanase under solid-state conditions; the precipitation of crude enzyme was suitable at 35% of ammonium sulfate under condition at 0°C and continually overnight at 4°C [24]. The inulinase of *X. campestris pv. phaseoli* KM24 was precipitated at 20-100% of ammonium sulfate for 16 h [19]. The requirement of salt concentration for precipitation is dependent on the properties and concentration of proteins present in the starting solution. However, the inulinase activity of *B. aquimaris* remained in the supernatant; this implied that the inulinase protein did not precipitate well as shown low specific activity in Table 1. On the other hand, the precipitated proteins contained some inulinase and some other proteins. This effect may cause from short time precipitation (only 1 h), resulting in less efficiency of binding between salt and inulinase. The protein may have thick hydration shells, leading to difficult precipitation.

Alcohol precipitation used ethanol as the precipitant at concentrations from 20-50% (Figure 1(b)); the results showed that 50% ethanol precipitation gave the highest enzyme activity at 338 U, the specific activity of 26.21 U/mg. Other studies using ethanol precipitation in exoinulinase from *K. marxianus* YS-1 [4]; the specific activity of 47 IU/mg was achieved with 85% (v/v) chilled ethanol. [16] purified inulin fructotransferase (DFA III-forming) from *A. aurescens* SK 8.001 by pre-chilled (-20°C) 60% (v/v) of ethanol at 4°C for 1 h, giving specific activity at 18.7 U/mg. The fructosyltransferase from *Rhodotorula* sp. was purified using ethanol precipitation up to 70% at 4°C, showing the specific activity of 0.2 U/mg (fructofuranosidase) and 0.5 U/mg (fructosyl-transferase) [25]. The ethanol has an effect of dielectric constant on the ability of water solubility depending on kind of proteins. The ethanol concentration is low; the fully enzyme precipitation might not occur. With high ethanol concentration, it can denature proteins, also may reduce enzyme activity and purification factor as containing some inhibitors [26].

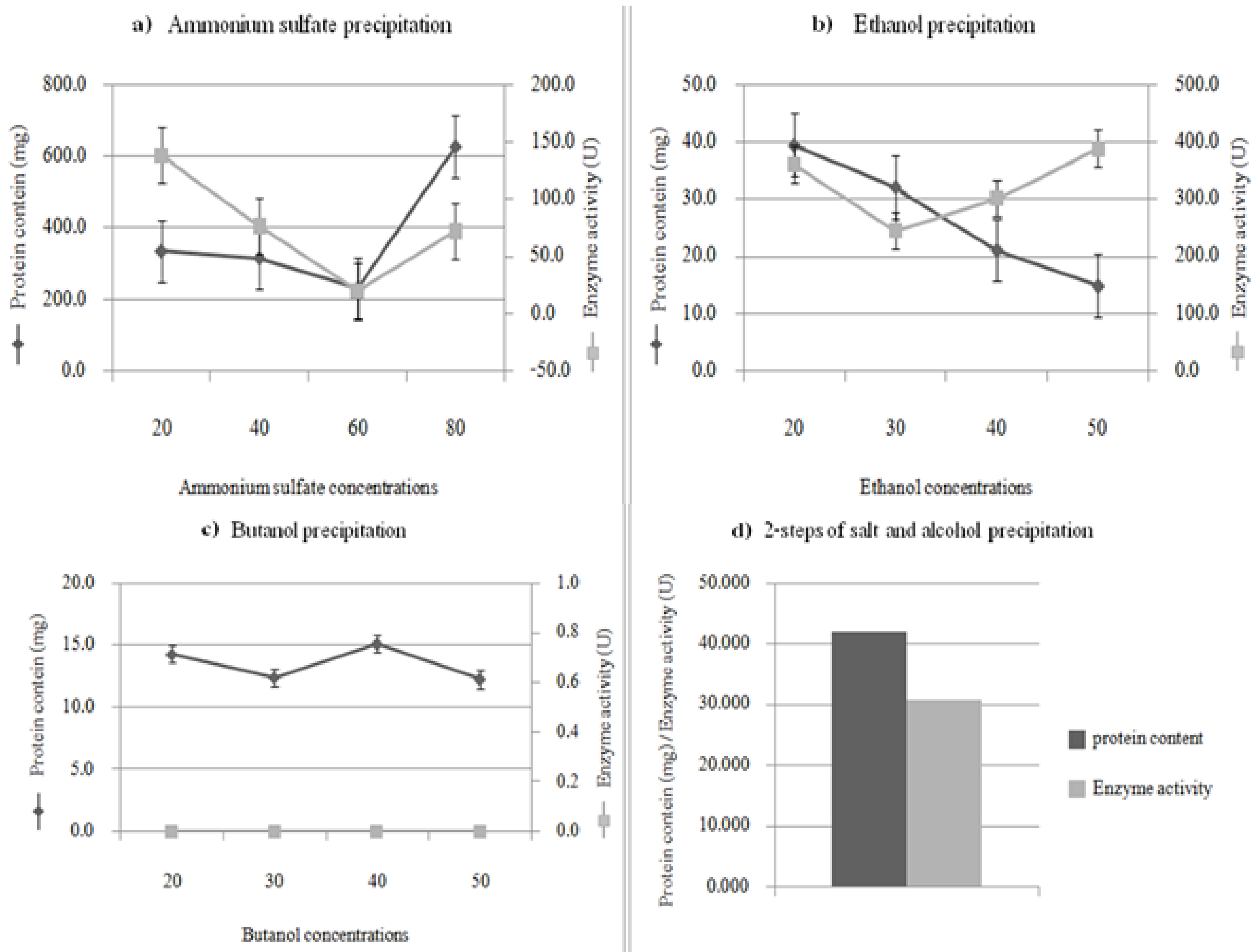

Fig. 1. Enzyme activity and protein content of inulinase in different precipitation methods.

In terms of butanol precipitation using t-butanol as the precipitant at concentrations from 20-50%, the results showed 40% of butanol giving the highest protein content (15 mg), but enzyme activity was not found (Figure 1(c)). The protein content and inulinase activity remained in the supernatant. This indicated that t-butanol required salts for protein precipitation. 2-steps of salt and alcohol (ammonium sulfate and t-butanol) showed enzyme activity at 31 U with 42 mg protein (Figure 1(d)). However, the protein contents of alcohol precipitation were very low and not enough for purification. This effect may cause by unfavorable interaction between polar groups of the protein and ethanol, leading to protein solubility. In contrast, the non-polar groups have favorable interactions with ethanol decrease protein solubility, causing protein precipitation [13]. The alcohols could inhibit protein functions by changing the conformation of protein which affected from loss of stability in hydrophobic interaction. Moreover, the long chain of hydrocarbons or high concentration of alcohol and high temperature are the major effects to damage protein structure [13].

Table 1 shows the crude enzyme of *B. aquimaris* with inulinase activity at 729 U and the protein content was 452 mg by Lowry's method, resulting in specific activity at 1.16 U/mg. The optimum condition of each precipitation method also shown in Table 1 to compare the suitable method for inulinase purification. The precipitation with 50% ethanol gave small amounts of protein content, but the specific activity, percentage of recovery and purification were the highest values. This implied that all protein contents that precipitated were inulinase. The effect of ethanol is not consistent for all proteins, and its effects are varied depending on temperature. Thus, the condition of ethanol precipitation should be developed to decrease the effects of ethanol on protein structure or functions, e.g., using pre-chilled ethanol, performing at low temperature (4°C).

TABLE 1
COMPARISON OF VARIOUS METHODS FOR INULINASE CONCENTRATION IN *BACILLUS AQUIMARIS*

Precipitation	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude enzyme	729	452	1.61	100	1
20% Ammonium sulfate	138	335	0.41	18.98	0.26
50% Ethanol	388	15	26.21	53.25	16.24
Butanol	0	0	0	0	0
2-steps of salt and alcohol	31	42	0.73	4.21	0.45

V. CONCLUSION

In this study, different precipitation methods of inulinase were investigated, including ammonium sulfate, ethanol, t-butanol, and 2-steps of salt and alcohol. The optimum condition of ammonium sulfate was 20% showing 19% recovery; however, this method seems to be not specific to inulinase as shown low specific activity. The suitable method of inulinase from *B. aquimaris* was 50% of ethanol precipitation showing high protein recovery and high purification. This method was specific to inulinase but it needs to study the optimum condition of procedure to achieve high protein contents. The modified method of ethanol precipitation may be used as a simple purification technique for inulinase from the endophytic bacteria, which may be applied in fructose-syrup production from inulin of Jerusalem artichoke.

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