Extraction of β 1,3-1,4 Glucanase from *Bacillus Subtilus*: Aqueous Two-Phase Extraction Versus Salt Precipitation

Pavithra M K S, Jayapriya A, Ismathroshan B M, Kokila S

Abstract: The activity of extracellular enzyme, β-1,3-1,4 glucanase extracted from Bacillus subtilis was validated on 0.5% starch agar plate The enzyme from the crude broth was extracted using two different methods i) Ammonium Sulphate Precipitation ii) Aqueous two-phase extraction. Ammonium sulphate precipitation was carried out using 65% of the salt. Aqueous two-phase extraction was carried out using PEG/ Ammonium Sulphate system using two different concentration of the salt as two different trials. From the study, it was found that extraction of the enzyme using aqueous two-phase process yields high enzyme concentration and increasing the PEG concentration increases the yield of the enzyme.

Key Words: β-1,3-1,4 glucanase, Ammonium Sulphate Precipitation, Aqueous Two-Phase Extraction, Starch Plate, Halo Zone

I. INTRODUCTION ABILITY

β-1,3-1,4-glucanases are industrially important enzymes with potential applications in animal feed production and brewing [1]. β-1,3-1,4-glucanases has the capability to break down glycosidic bonds present in β-glucan. These enzymes have the ability to degrade fungal cell walls and acts against pathogenic fungi [2]. Industries use crude enzymes isolated from *Bacillus* sp.

The presence of other enzymes co-produced such as amylases and proteases leads to difficulties in the purification processes [3]. The partitioning and purification of the enzymes depends on the charge, size, hydrophobicity and surface properties [4]. Extensive studies on the *Bacillus* species shows the production of a wide range of extracellular polysaccharide hydrolyzing enzymes [5]. Partitioning of the enzymes using Aqueous Two-Phase Extraction Process has been proven to be a authoritative strategy for separation and purification of protein molecules from the crude extracts. The methodology uses polar solutions such as water-soluble polymers or polymer - salt system [6]. It has been studied that MW and concentration of Polyethylene glycol have significant effects on partition and extraction of the enzyme [7]. *Bacillus* sp. grown on LB broth containing starch produces β-1,3-1,4 glucanase that appeared to be homogenous on the Polyacrylamide gel [8]. The present study focused on the comparison of β-1,3-1,4 glucanase (β-1,3-1,4-G) extraction from *Bacillus subtilis* using Ammonium sulphate precipitation and Aqueous two-phase extraction.

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II. MATERIALS AND METHODS

A. *Strain and growth conditions*

Bacillus subtilis MTCC 441 was procured from IMTECH, Chandigarh, India. The lyophilized culture was inoculated in Hiveg nutrient substrate and kept at 37°C for 24 h. Further sub-culturing was carried out by transferring the culture into nutrient broth at 1% (v/v). The cultures were maintained by subculturing aseptically, at fortnight intervals [9].

B. *Extracellular enzyme assay*

The ability of the enzyme present in the broth to hydrolyze starch was evaluated using agar diffusion method by using 0.5% of the substrate. Small wells were made on the agar plate and the wells were loaded with 100µl of the supernatant of the two-day cultured broth and incubated for 24 hours at 37˚C. The plates were blemished using Iodine solution for the development of halo zones [10].

C. *Purification of β-1,3-1,4-G*

The crude broth was then centrifuged at 4°C and 10,000 rpm for ten minutes for the supernatant. The supernatant was divided into two fractions, sample 1 and sample 2 [11].

D. *Ammonium sulphate precipitation*

Overnight grown *B.subtilis* cultures was filtered through filter paper (Whatman no. 1) and centrifuged at 6,000g for 10 min. 80% saturated ammonium sulfate was used to precipitate the supernatant. The precipitate was resuspended in 5 ml sterile dH_2O and washed. The precipitate was dialyzed at pH 5.5 using 50 mM potassium acetate buffer. The dialyzed fraction containing the crude enzyme is obtained at a final volume of 10 ml [12].

E. *Aqueous two-phase extraction*

Extraction was carried out using phase systems of PEG (20%, w/w) and Ammonium sulphate (10% and 30%, w/w). The concentration of the PEG and Ammonium sulphate was determined from the bimodal curve (Figure 1). The sample 2 was divided into two fractions. To one fraction (sample I), 10% ammonium sulphate salt and 20% PEG was added to get 100% (w/w) sample and mixed well and allowed to separate into two layers.

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To another fraction (sample II), 30% ammonium sulphate salt and 20% PEG was added to get 100% (w/w) sample and mixed well and allowed to separate into two layers. The salt phase from both the samples was dialyzed using 20mM tris buffer [13].

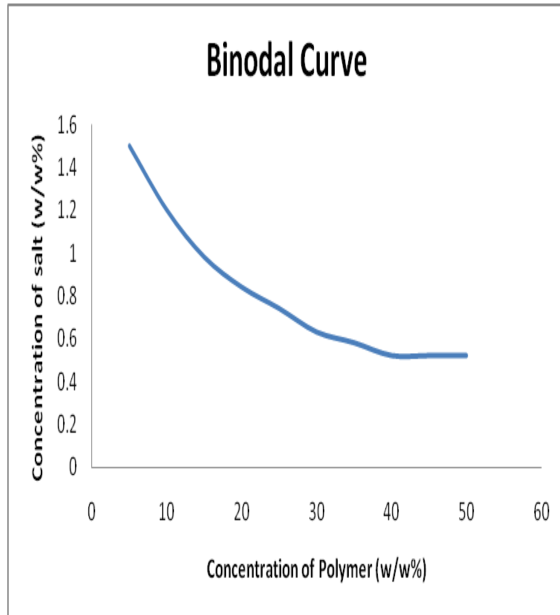

Figure 1: Bi-nodal curve for PEG and Ammonium Sulphate system

F. Extracellular enzyme assay

Extracellular enzyme assay was carried out to find the localization of the enzyme in the two phases using 100μl sample from each phase using agar diffusion method on the starch plate [13]. The activity of *β-1,3-1,4-G* was validated by incubating 10% of the enzyme with 90% of 1% carboxy-methylcellulose in 100 mM Tris-HCl at 50˚C, pH 7.0 for 40 min. The reducing sugar concentration was computed using standard procedures [14].

G. Protein determination

The assay rests on the ability of protein molecules to bind coomassie brilliant blue G250 using standard BSA [15].

H. Validation of purity

PAGE was carried out to validate the purity of the enzyme obtained by both the methods [16].

III. RESULTS AND DISCUSSION

A. Extracellular Enzyme Assay for the crude extract

The formation of clear ring near the well indicates the presence of glucanase in the crude extract as shown in figure 2. The clear zones formed on the starch plate confirms the hydrolysis of β-glucan indicating β-glucanase production by the bacterial cell [10].

Figure 2: Glucanase activity of the crude enzyme

B. Extracellular Enzyme assay after Ammonium Sulphate Precipitation

The activity of enzyme on the starch plate before and after dialysis was represented in figure 3. Well 1 and 4 are loaded with crude enzyme after dialysis and well 2 and 3 are the samples after dialysis and purification. Increase in activity of enzyme in the combined process is clearly illustrated with increase in the diameter of the halo zone formed.

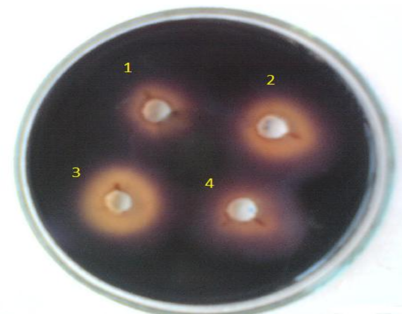

Figure 3: Glucanase activity on starch plate

C. Biochemical Test For glucanase

The concentration of enzyme obtained after dialysis is determined using Barfoed's method. The concentration of glucanase obtained was 14.52 µg/ml. Extracellular Enzyme assay after aqueous two-phase extraction

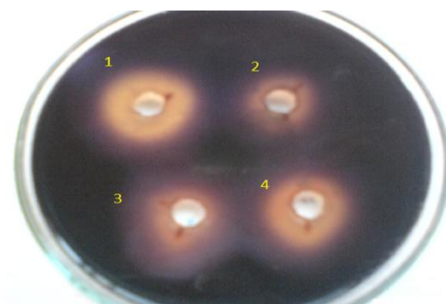

Figure 4: Glucanase activity by Aqueous Two-Phase System

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Figure 4 represents the activity of glucanase after purification by the two trials. The zone 1 and 4 is produced by the enzyme using Trial 1 system whereas zone 2 and 3 are formed by the sample in the organic phase using Trial 2. The amount of enzyme in the aqueous phases is very less for the formation of halo zones which confirms that the enzyme is in the organic phase. Zone 2 and 3 clearly implies that Trial 2 can be used for purification of the enzyme with profound activity.

D. Assay for Glucanase

The quantity of β-1,3-1,4-G in the organic and aqueous phases after the extraction phases determined using Bradfoed's method is given in table 1.

Sample	Phase	Concentration (µg/ml)
I	Organic Phase	19.27
	Aqueous Phase	0.093
II	Organic Phase	21.58
	Aqueous Phase	0.033

Table:1 Concentration of protein

E. Validation of purity of the enzyme

The presence of the molecular band corresponding to 29 kDA confirms the presence of the enzyme in the two methods being done [17]. The Polyacrylamide gel clearly represents the efficiency of two-phase system for the downstream of β-1,3-1,4-glucanases. The gel image indicates the advantage of using the ammonium sulphate precipitation and aqueous two-phase extraction for *β-1,3-1,4-G* purification

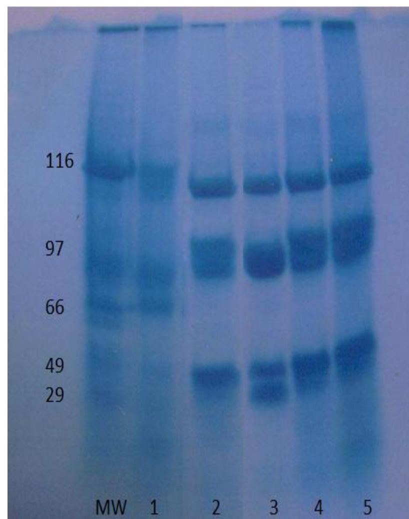

Figure 5: Polyacrylamide Gel containing the enzyme

(1: Crude extract; 2: Enzyme from Ammonium sulphate Precipitation; 3: Combined Ammonium sulphate Precipitation and Aqueous two phase extraction; 4: Trial 1 of Aqueous two phase Extraction; 5: Trial 2 of Aqueous two phase extraction)

IV. CONCLUSION

The current findings show that combined ammonium sulphate precipitation and aqueous two phase extraction shall be carried out for *β-1,3-1,4-G* purification. Further downstream processing shall be done to enhance the activity of the enzyme.

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