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PARTIAL PURIFICATION OF CELLULASE PRODUCED BY CELLULOMONAS SPP AND DETERMINATION OF ITS ENZYME ACTIVITY AND ENZYME KINETIC PARAMETERS

Lahudkar Dnyaneshwari, Ghanwat Kritika

M.Sc. (Microbiology), M.Sc. (Microbiology)

Corresponding Author- Prof. Pansare Ragini

Department of Microbiology,

New Arts, Commerce and Science College, Ahmednagar, India

Abstract: Cellulose degrading bacteria was isolated and was purified. Cellulase extraction was carried out and was optimized. In this study, enzyme kinetic parameters were determined. The Km of the enzyme is 12.04 µmoles whereas Vmax is 5.88 µmoles/ml/min. The enzyme was then further partially purified by ammonium sulphate precipitation and dialysis method. In ammonium nitrate precipitation, the enzyme showed maximum precipitation at 60% of ammonium sulphate. The protein content was found to be 518 µg/ml. The enzyme purified by ammonium sulphate precipitation showed the yield of 68.91%. The specific activity was increased from 0.74 U/mg to 1.274 U/mg. The purification factor was 1.722-fold. After dialysis, the concentration was increased from 0.279 µg/ml to 0.3906 µg/ml, thus, the enzyme was purified. The bacterial isolate was then applied to some of the leading industrial biproducts like sugarcane bagasse, paper pulp and sugarcane molasses, which are the biproducts of sugarcane mills, paper industry and sugar industries respectively.

Index Terms: Extraction, parameters, precipitation, dialysis, applied.

INTRODUCTION

Cellulose is the most abundant biomass on earth [22]. This abundancy makes cellulose an attractive raw material for producing many industrial important commodity products [10]. So, cellulase become the most important enzyme of today in industrial biotechnology [14]. About 15% of the global market of industrial enzymes consist of cellulase. This creates a need of new ingredients of cellulase production [29].

Cellulase is produced by every living body that utilizes cellulose. In industries, it can be produced by number of microorganisms including bacteria and fungi [8]. They have different mechanisms that degrades various forms of cellulose such as lignocelluloses and hemicelluloses [29]. Cellulolytic organisms are

ubiquitous in nature [21]. Bacteria are commonly used for the purpose of high production [29]. They have high growth rate than fungi and also have a good potential for production [22]. Some of them include species of *Clostridium*, *Butyrivibrio*, *Bacteroides*, *Ruminococcus*, *Cellulomonas* etc. [10]. Bacterial enzymes are known to be more stable to the industrial processes like bioconversion, which further increase the efficacy of fermentation [20]. This enzyme is used as production material in industry [18]. Cellulolytic bacteria are isolated from various niche including composts, soils, decaying plants, feces of insects and birds and so on [20]. One of the easily approachable sources is of the ruminants.

The importance of these enzyme is highly recognized in every industry producing cellulose as a biproduct. They also dominate the waste of agricultural sector [29]. These types of wastes are needed to be managed in order to grow a sustainable environment free from environmental pollutants [24]. These wastes generally comprise of lignocellulosic waste [18]. Conversion of these cellulosic materials into chemicals and fermentable sugars offer a great option to serve the environment [24]. These are extremely resourceful and cost effective. Cellulose degrading bacteria was isolated and was purified. Cellulase extraction was carried out and was optimized. In this study, enzyme kinetic parameters were determined

MATERIALS AND METHODS

1.Determination of enzyme kinetic parameters

Determination of Km and Vmax Initial reaction rate of cellulase hydrolysis was determined by varying its substrate concentration in the range of 2 to 20 µmoles /ml in standard cellulase assay and the double reciprocal plot (Lineweaver and Burk's plot) was drawn. The kinetic parameters Km and Vmax were estimated from this plot.

Table 1- Readings obtained for Lineweaver and Burk's Plot

Sr. No	Substrate concentration (µmoles/mL)	1/ [S] (mL/µmoles)	O.D at 540 nm	[V] (µmoles/ml/min)	1/ [V] (µmoles/min/ml)
1	2	0.5	-0.020	-2	-0.5
2	4	0.25	-0.021	-2.1	-0.476
3	6	0.166	-0.024	-2.4	-0.416
4	8	0.125	0.025	2.5	0.4
5	10	0.1	0.027	2.7	0.370
6	12	0.083	0.033	3.3	0.303
7	14	0.071	0.036	3.6	0.277
8	16	0.062	0.040	4	0.25
9	18	0.055	0.046	4.6	0.217
10	20	0.05	0.05	5	0.2

2.Enzyme activity of crude cellulase-

Cellulase activity of the isolate was assessed by DNS method by measuring the amount of reducing sugar released during enzymatic reaction by Dinitrosalicylic acid. The reaction mixture was composed of 1 ml crude enzyme and 1 ml of DNSA. After boiling the mixture for 10 min, 8 ml of distilled water was added. The colour of reducing sugar developed was measured spectrophotometrically at 540 nm wavelengths and the activity was measured and enzyme units were calculated by using standard graph of glucose. Cellulase activity was expressed as a units of glucose released per ml per min. The experiments were performed in triplicate (n=3).

3. Partial purification of cellulase enzyme

The clear supernatant obtained after centrifugation was subjected to partial purification by ammonium sulphate [$(NH_4)_2SO_4$]. 20 ml of the supernatant was taken and was saturated with 10% of ammonium sulphate, which was incubated at 4˚C overnight and observed for precipitation. Since no precipitation was observed, a new batch of 20 ml supernatant was saturated with 20% ammonium sulphate and incubated. These tests were carried out until the precipitation was observed. The flask with 60% of saturation showed precipitation. This protein precipitate obtained by centrifugation at 5,000 rpm for 20 min at 4ºC was dissolved in 5 ml. 0.1 M sodium phosphate buffer of pH 7.The quantity protein in this precipitate was estimated by Folin-Lowry method using bovine serum albumin (BSA) as standard. Also, the parameters like yield, specific activity and purification factor were calculated. The precipitate was also dialysed extensively against citrate buffer of pH 4.3 at room temperature for 1h. The dialysed sample was considered as partially purified.

4. Applications in sugar industries, paper industries and sugarcane mills

To demonstrate the utilisation of molasses. Paper pulp and sugarcane bagasse biproducts of sugar industry, paper industry and sugarcane mills respectively, minimal broth with respective biproducts were prepared. The broths were autoclaved and inoculated with the culture to demonstrate utilisation of the biproducts as cellulose substrate. The broths were further incubated for 24h and the cellulose reduction was assayed by DNS method.

RESULTS

1.Determination of enzyme kinetic parameters

Km and Vmax of the enzyme was determined by extrapolating the reciprocal graph (Lineweaver and Burk's plot) of substrate concentration verses velocity. Velocity was determined with the help of standard graph of glucose of 20 µmoles/ml. The value of Km was found to be 12.04 µmoles and that of Vmax was 5.88 µmoles/ml/min

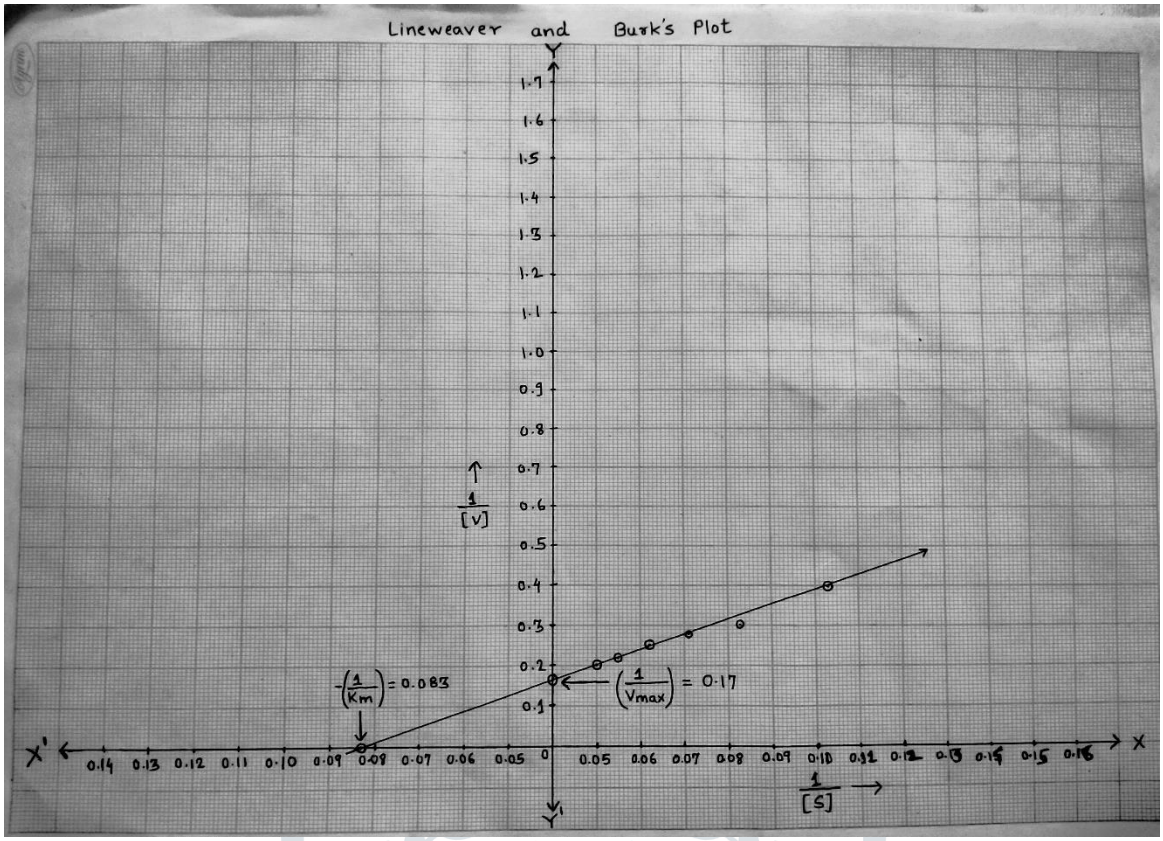

Fig 1: Lineweaver and Burk's plot for determination of Km and Vmax

2.Enzyme activity of crude cellulase-

The cellulase activity was determined of the crude cellulase that was extracted. The O.D obtained was extrapolated on the standard graph of glucose of concentration 1000µg/ml. After the extrapolation and calculation by the formula-

$$\text{Enzyme units} = \frac{\mu mole\ of\ product\ formed \times Volume\ of\ enzyme}{Reaction\ time}$$

The enzyme units of the crude cellulase were found to be 0.162 units.

3.Partial purification of cellulase enzyme

Partial purification was done in two steps. The enzyme was partially purified by 60% ammonium sulphate precipitation. Specific activity of enzyme was increased from 0.74 U/mg to 1.274 U/mg. The purification factor was 1.722-fold with the yield of 68.91%. The concentration of the dialysed sample increased from 0.279 to 0.390µg/mL after 1h.

4. Applications in sugar industries, paper industries and sugarcane mills

The O.D of all the samples (paper pulp, sugarcane bagasse, solid and liquid molasses) were increased after incubation of 24h. This indicated that the bacteria readily utilize cellulose as its substrate for its

metabolism as a carbon source. The bacteria can further be subjected to fermentation for large scale production of the enzyme.

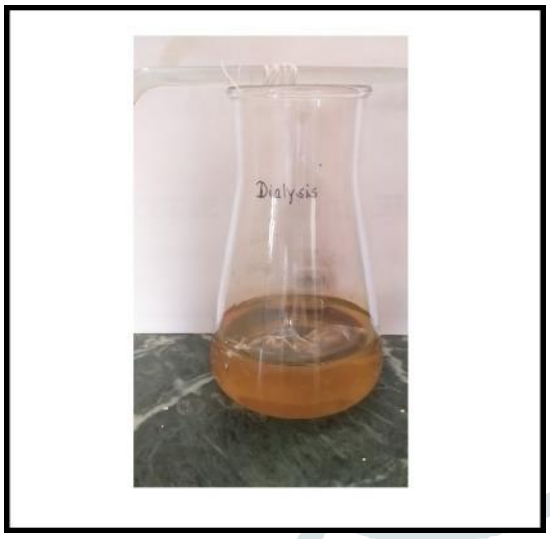

Fig 2: Dialysis

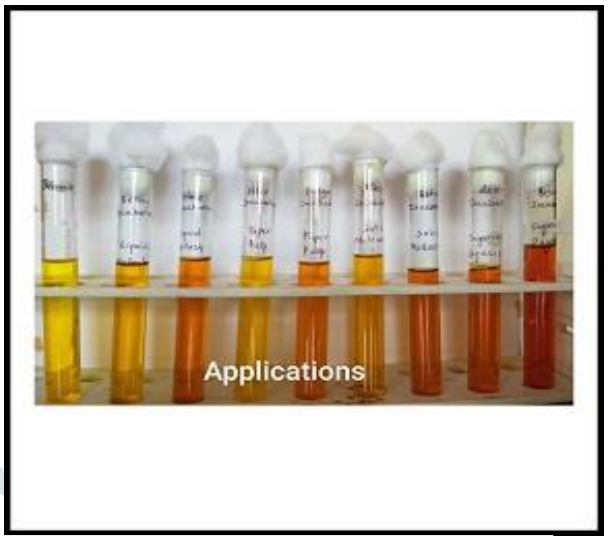

Fig 3: Applications

DISCUSSION

Earlier reports on the microbial cellulase indicate that Vmax of cellulase extracted from A. Niger was found to be 9.26 U/mL while the Km was 0.23 mg/ml (A.O Sulyman et.al.,2020). The ammonium sulphate precipitation keeps the high salt concentration crude enzyme away from nonprotein and was typically included in purification protocols (Wei Wang et.al.,2007). The bacterial cellulase was precipitated at 60% ammonium sulphate precipitation (Ashok Shinde et.al.,2020). The enzyme was purified to 68.12-fold with yield of 3.87% and specific activity of 484.3 U//mg (A.O Sulyman et.al.,2020). The agricultural by- products like cereal straws, rye, barley, oats etc. were checked for the activity of cellulase (Agnieszka Wita et.al.,2019)

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