EFFECT OF MEDIA COMPOSITION AND CULTURE CONDITION ON PULLULANASE ENZYME FROM EXTREMOPHILE BACTERIAL SPECIES

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Abstract

Pullulanase is of great significance due to its wide area of potential application. Pullulanase is widely used in industries in the saccharification of starch. It converts starch into glucose and maltose which are used in the production of glucose syrup more efficiently. In this present study the enzyme was isolated from the extremophilic strains of \textit{Streptococci} isolated from extreme conditions. Pullulanase enzyme 98 KDa was successfully produced and purified by the isolated strains. In this process to study enzyme activity the enzyme substrate reaction was performed based on various parameters temperature 50°C, time 72 hours, and pH 6. As it is clearly stated after the detail analysis of all parameters that the pullulanase isolated in the present study is very stable and active at different parameters. The Pullulanase activity of Gel filtration column chromatography of precipitated protein by ammonium sulphate gave rise 130.66 IU pullulanase activity which was greater than the 0-30% (98.0 IU) and 70-90% (130.66 IU). And thus it can be used in various industrial processes where normal mesophilic enzymes cannot be applied.

Keywords: Pullulanase Enzyme, Optimization physical parameter, Media composition

1. Introduction

The roles of enzymes in many processes have been known for long time. Their existence was associated with the history of ancient Greece where they were using enzyme from microorganism in baking, brewing, alcohol production, cheese making etc. With better knowledge and purification of enzyme a number of applications has increased many folds, and with the availability of the enzyme a number of new possibilities for industrial process have emerged. Pullulanase is one such industrially useful enzyme used in the starch processing industries. Pullulanase belongs to \( \alpha \)-amylase class of enzymes which is an extracellular carbohydrase which was first discovered by Bender and Wallenfels in 1961 from mesophilic organism \textit{Klebsiella pneumoniae} (formerly known as \textit{Aerobactor aerogenes} or \textit{Klebsiella aerogenes})\textsuperscript{2-3,4}. Pullulanase are also called de-branching enzyme which hydrolyze the extracellular yeast, polysaccharide and pullulan. It converts pullulan into the trisaccharides, maltotriose. It consists of repeating units of \( \alpha \)-maltotriose joined “head to tail” by 1, 6-bonds \textit{Ling et al.}\textsuperscript{2} Pullulanase specifically attack on \( \alpha \)-1, 6-glycosidic linkage and it also attacks on \( \alpha \)-1, 4-glycosidic linkage with other residues, these properties have made pullulanase a useful agent in structural studies of oligosaccharide and polysaccharide\textsuperscript{3}

There is an acute needs to produce this enzyme from species of extremophiles that can be able to produced pullulanase under extreme conditions required during commercial productions. The aim of this research work was to study the effects of different concentrations of carbon, nitrogen, minerals, metal ions, additives and substrate for the optimum production of pullulanase from extremophiles. In addition, the effect of various fermentation parameters such as pH, temperature and incubation time on parameters of pullulanase fermentation in synthetic medium was studied.

2. Materials and Methods

2.1 Collection of soil sample: Collection of soil sample from various sites like Jaisalmer (Rajasthan), Bhopal, Barwani, and Khandwa (M.P.) sample were transferred into sterile 50 ml vials that were immediately sealed with rubber stopper and were transported to the laboratory at 4\( ^\circ \) C in thermic containers

2.2 Isolation and culture condition: Soil is a collective source of many types of organisms. For our work we need to screen out only extremophilic organisms to be used as a source of enzyme pullulanase. Subsequently following steps were performed to screen out extremophilic organisms.

2.3 Screening of extremophiles:

2.3.1 Dry heat treatment: Dry heat shock treatment of sample was conducted for screening
of extremophiles. For dry heat treatment 10 gm of soil samples were taken in petridish. Incubated at 65°C in hot air oven for 5 min. Samples were taken out from hot air oven and allowed to cool. Again the plate was placed in hot air oven. This process was repeated 4 times. After cooling the sample were used for further screening.

2.3.2 Serial dilution: Serial dilutions of soil samples after the dry heat shock treatment was done for the isolation of extremophile from the soil sample collected from various extreme environments. 1 gm of soil sample was dissolved in 10 ml double distilled water. 1 ml of this solution was then taken and the volume was made up to 10 ml by double distilled water; the dilution will then be 10⁻¹. Then 1 ml of dilution 10⁻¹ was taken and the volume was made up to 10 ml by DDW, this will be dilution 10⁻². Dilutions were prepared up to 10⁻¹⁰ by following same process.

2.3.3 Wet heat treatment: The spores of mesophiles present in the soil samples that have survived dry heat treatment get inhibited by wet heat treatment. The wet heat treatment ensures the survival of only extremophiles as the pressure of steam in case of wet heat treatment has higher penetration power. For wet heat treatment 10 ml of soil suspension (10⁻⁸) was kept at 65 °C in autoclave for 3 min. The sample was then taken out from autoclave and allowed to cool. Again the plate was put in autoclave. This process was repeated 4 times. After cooling the sample were used for pH screening.

2.3.4 pH shock treatment of sample for screening of extremophiles: After heat shock treatment only temperature tolerant organisms are obtained but in order to isolate pH tolerant extremophiles, pH shock treatment of the samples was conducted. Two sets of 5 tubes each, containing 10 ml of nutrient broth media were prepared. The pH was adjusted of one of the sets to 8 to 12 with addition of NaOH and of another set to 4 to 8 with addition of HCl. Each tube was inoculated with 0.1 ml of suspension (10⁻⁸). Tubes were then incubated at 65 °C for 24 hours. After 24 hours the suspension was used for further processing. By the application of above mentioned screening methods the chances of isolating temperature and pH tolerant extremophiles which can be used to produce more stable enzymes increased. After the screening of extremophiles from the soil samples they were characterized by following experiments.

2.4 Parameters of colony characteristics: Colony count and morphological study on nutrient agar plate as observed by naked eyes based on different parameters like: Size, Shape, Edge, Elevation, Texture, Pigmentation and Opacity were conducted for the interpretation of colonial characteristics. Gram staining was done for the identification of gram negative or positive bacteria on the basis of Morphology of cells, Colour of cells, and Colour of background.

2.5 Screening of pullulanase enzyme producing strains from mix culture: Suspensions prepared after screening of extremophiles further screened for the isolation of strains producing pullulanase by inoculating in differential media based on their biochemical reactions. The plate assay was performed using agar plates enriched with pullulan. For this purpose 0.1 ml suspension was spread pullulan agar media and then incubated on gradually increasing order of temperature 25°C-55°C and pH 4-10. The pullulan agar media used for the differentiation and isolation of pullulanase producing strain.

2.6 Morphological and Biochemical Characterization for Identification of pullulanase producing strain: The morphological characteristics were observed after gram staining and a further characterization was performed by using certain biochemical tests as mention. Various biochemical tests for a broad classification of the bacteria according to (Bergey’s Manual). Sugar fermentation medium (peptone water sugar), Catalase test, Coagulase test, Milk agar and Haemolysis test.

2.7 Production of pullulanase: Production medium (pH 7.4) Modified Mineral Czapek Medium⁴ (Brandt et al., 1976), the culture medium consisted (w/v) of Peptone 0.8%, K₂HPO₄ 0.1%, NaNO₃ 0.5%, MgSO₄ .7H₂O 0.05%, KCl 0.05%, Maltose 0.5% gm, and Distilled Water 100ml. the pH of all media was adjusted to 5.5 with 1 M HCl (One factor at a time method). The production media was sterilized by autoclaving at 121°C and 15 lb/inch square pressure for 15 minutes. The inoculums were prepared by germinating the microorganism from the solid agar medium in 250 ml flask containing 150 ml of selected production medium and incubated at 40°C for 24 hours. After incubation of the cultures at 40°C for 72 hrs, the cells were removed from the culture medium by centrifugation at 10,000 rpm for 15 min at 4°C. The supernatant was used for further purification.
The supernatant was collected and precipitated by ammonium sulphate method. The supernatant (100 ml for production batch) was treated with \((\text{NH}_4)_2\text{SO}_4\) at 40%, 60% and 80% saturation and the flask was continuously stirred for overnight precipitation. The precipitation was carried out at 4°C with gentle stirring on a magnetic stirrer. The precipitate formed was collected by centrifugation at 12,000 rpm for 20 minutes and the pellets were suspended and dissolved in 0.02 M sodium phosphate buffer, pH 6.9 and dialyzed against the same buffer for 24 h at 4°C.

2.8 Purification of enzyme: This was the extraction and purification of desired enzyme from the production medium. The downstream processing of pullulanase was done by following steps.

2.8.1 Cell disruption: Cell disruption was the first step in the process of enzyme purification. As in present study, it was unknown that whether the enzyme was secreted endogenously or exogenously, therefore cell disruption technique was applied so as to release whole enzymes in suspension even if it was endogenous in nature. Cell disruption was done by Glass bead homogenizer after freeze thaw lysis.

2.8.2 Separation by Chromatography: All chromatographic steps were performed at 4°C. The dialysed enzyme preparation was applied to a DEAE-sepharose CL with 0.02 M sodium phosphate buffer, pH 6.9, the column was washed with the same buffer at a flow rate of 1.8 ml min\(^{-1}\) fraction exhibiting pullulanase activity were pooled and concentrate by freeze drying. Sample were dissolved in a minimal volume of 0.02 M sodium phosphate buffer, pH 6.9 and 4°C with tow buffer change.

For further purification of the enzyme gel filtration chromatography was performed onto a superdex 75 with 0.02 M sodium phosphate buffer. Elution at a flow rate of 0.5 ml min\(^{-1}\) was performed using the same buffer. The active fraction pooled and concentration by ultra filtration was used as the purified enzyme for further analysis. The purity and molecular mass of the enzyme were characterization and determined using SDS-PAGE.

2.8.3 Assay of pullulanase activity: Enzyme activity was determined by measuring the enzymatic release of reducing sugar from pullulan. In this assay, 0.5 ml of enzyme sample was added into 0.5 ml of 1% (w/v) pullulan in 0.02 M sodium phosphate buffer, pH 6.9. The reaction mixture was incubated at 40°C for 30 min and immediately allowed to cool. The reaction was terminated by adding by 3 ml of DNSA solution followed by heating for 10 minutes. The OD was taken at 540 nm\(^6\).

The amount of reducing sugar released at the end of the reaction was determined by Dinitro Salicylic Acid (DNSA) method. Sample blank was used to correct for the non-enzymatic release of reducing sugar. Standard curve was made by taking OD of sample containing 1 ml of glucose solution in the range of 50 micro moles to 400 micro moles, 0.5 ml of pullulan in water and 0.5 ml pullulan in phosphate buffer. One unit of pullulanase activity is defined as the amount of enzyme required to produce 1 µ mol reducing sugar (equivalent to glucose) min\(^{-1}\) under the assay condition\(^5\).

2.9 Physical parameters: The factors such as pH, temperature and incubation period, affected the production of pullulanase, and these were optimized by varying parameter at a time. The experiment was conducted in 250 ml flask containing production medium. After sterilization by autoclaving the flask were cooled and inoculated with culture and maintained under various operational conditions separately.

2.9.1 pH: Every microorganism has a particular pH at which it grows the best this pH called as the optimum pH, The pH optimum for the enzyme activity was studied over a range from pH 4 -10 with pullulan as a substrate. The pH stability of the enzyme was determined by incubating the enzyme in buffers with different pH for 24 hrs and incubated at 30°C. The following buffer systems were used under various operational conditions separately such as pH 4, 5, 6, 7, 8, 9 and 10\(^5\).\(^7\).

2.9.2 Temperature: Every microorganism has grown a particular temperature at which it grows the best this temperature called as the optimum temperature. The incubation temperature directly affected the enzymatic activity of the cell and thus the overall metabolism is disturbed if there is any fluctuation in the incubation temperature separately such as Temperature 25°C, 30°C, 35°C, 40°C, 45°C, 50°C and 55°C\(^5\).\(^7\).

2.9.3 Incubation Time: Pullulanase activity was determined at different time intervals of incubation such as 24 hrs, 48 hrs, 72 hrs and 96 hrs etc. The optimum pH required for the pullulanase production and growth of microorganism was determined by adjusting the pH of the fermentation medium at pH ranges.
between 4 to 10 separately in 250 ml conical flasks and incubated at 30°C for 7 days in shaker incubator 200 rpm. Culture were studied every 24 hrs.

2.9.4 Media Composition: Media composition play an important role in the growth and overall growth of microorganisms, different ingredients of media such as carbon sources, nitrogen sources, minerals, metal ions, polyols and additives have different effects on growth and activity of microorganisms, thus these parameters needs to be optimized to obtain the optimal growth of microorganism and thus will lead to the better production yields of the desired enzyme.

3. RESULTS AND DISCUSSION

3.1 Characterization of pullulanase producing colonies: The first step for the analysis of unknown bacteria was the observations of colonial characteristics result are described, By analysing the colonial characteristics on nutrient agar plate, the colonies of desired enzyme producing strain were found as small, circular pin point and semitransparent.

3.2 Gram staining and Biochemical characterization: Further characterization was done based on the gram reaction as in the present study the strain is gram positive so that following tests were performed for the identification of unknown bacteria. After the gram staining the pullulanase producing strain was found as a gram positive organism. Further characterization was done by performing various biochemical tests after study the gram staining. By analyzing all biochemical tests performed for the characterization, the strain was identified as *Streptococci* as it shown beta haemolysis on blood agar media. The strain showed all sugar utilization positive with starch hydrolysis with motility and catalase negative and coagulase test positive which amide the confirmation of *Streptococci*.

The blood haemolysis test was performed to confirm the presence of *Streptococci*. The streptokinase enzyme produced by *Streptococci* degrades the blood cells present in media which can be seen as clear zone surrounding the colonies of *Streptococcus* spp. which amide the confirmation of *Streptococci* spp.

3.3 Conformation of pullulanase production: This is indication of pullulanase production. The plates were then flooded with iodine to observe clear zones easily.

![Beta haemolysis on blood agar plate confirms the presence of *Streptococci*.](image1)

![Colonies of *Streptococci* showing clear zone due to production of pullulanase](image2)

Plate A: indicate clear zone due to hydrolysis of pullulan by pullulanase production.
Plate B: indicate after flooded by iodine solution visualized zone clearly.

The clear zone shown by *Streptococci* is due to starch hydrolysis which can be clearly visualised by flooding the plate with iodine solution. Due to reaction with iodine the starch present in surrounding the colonies gives blue colour while the clear zone indicates the starch hydrolysis by pullulanase production.

3.4 Growth analysis of microorganism: Growth analysis was done by using dry weight analysis after 1, 2, 3, 4, 5, 6, and 7 days. From the result it was determined that log phase last up to 5 days.
The growth of *Streptococci* was checked up to 7 days (in reference to biomass in gm). The growth was continuing increased up to 7th day, but very fast increased in starting 4 days and after 4 day slowly decreased.

### 3.5 Media optimization on different parameter:

Optimization of different parameter, like as temperature, pH and time were identified as a most influential among the physical parameter, beside this carbon and nitrogen sources were also important.

#### 3.5.1 Effect of the incubation time on the activity of pullulanase:

The growth of microorganism and their enzymatic activities were estimated during incubation period which extended up to 72 hours. The result shows in the graph 2. The biosynthesis of extra cellular enzyme increased almost linearly until the stationary phase of growth was attained. Growth as well as pullulanase activity reached maximum value after 72 hours of incubation.

#### 3.5.2 Effect of the Different pH on the activity of pullulanase:

The activity of pullulanase was checked on various pH (4 to 10). The maximum enzyme activity was observed at pH 6 with respect to increase in pH the enzyme activity gets decline.

#### 3.5.3 Effect of the Different temperature on the activity of pullulanase:

Temperature is one of the most important parameter governing growths of organism and enzyme biosynthesis. The enzyme production pattern shows a gradual increase in the enzymatic activity giving maximum production at 50°C. At higher temperature due to the production of the large amount of the metabolic heat, the fermenting substrate temperature shoots up, there by inhibiting microbial growth and enzyme production.

#### 3.5.4 Effect of the Different carbon source on the activity of pullulanase:

The effect of different carbon sources on the production of pullulanase was studied using the basal culture medium supplemented with 2% of carbon enzyme production is highest at the initial pH 6-7. It reduced with the increase in pH as described in the graph 3. The pH value was adjusted by NaOH and HCl as required.
sources. The culture containing 2% of each carbon source at a time was incubated and tested for enzymatic activity. Among; glucose, galactose, lactose, maltose, pullulan, sucrose and starch were used 2% at a time. Maltose was best to enhance the pullulanase activity (graph 5) showing highest pullulanase activity (109.6 IU) as compare to other carbon sources. The highest pullulanase activity may be due to inducible nature of maltose being hydrolytic product of starch.

Graph 5: Graph showing activity of enzyme with respect to different carbon sources.
Various carbon sources were checked as media component and pullulan was observed with best enzyme activity, while maltose, sucrose and glucose were showing gradually decreasing but significant enzyme activity.

3.5.5 Effect of various nitrogen sources on the activity of pullulanase: The effect of different nitrogen sources on the production of pullulanase was studied using the basal culture medium supplemented with 0.5% of nitrogen sources. Among the four organic nitrogen sources, peptone had most influence on pullulanase production (98.0 IU) than other (Graph 6). This may be low molecular weight of peptone, which can be easily degraded and can be absorbed.

Graph 6: Graph showing activity of enzyme with respect to different nitrogen sources.
Different nitrogen sources were checked for enzyme activity when the peptone given the maximum activity.

3.5.6 Effect of various minerals on the activity of pullulanase enzyme: To investigate the effect of minerals, the basal medium devoid of any mineral sources was supplemented with 0.5% of different minerals. It can be seen that only Dipotassium phosphate enhanced the pullulanase production.

Graph 7: Graph showing activity of enzyme with respect to different minerals sources.
It was also observed that in the medium with MgSO₄, pullulanase activity was stable with other minerals sources. Also unlike earlier reports, pullulanase production was not enhancing in presence of sodium and zinc chloride. Although the decrease in pullulanase production with incorporation of ferrous salts. The activity against mineral supplements was checked with different mineral salts added in production media. The potassium salt was observed maximum enzyme activity.

3.5.7 Effect of various metal ions on the activity of pullulanase enzyme: The inhibition effect of different metal ions on the activity of pullulanase from Streptococcus is shown in the graph 8. The pullulanase activity was inhibited strongly by Co²⁺, Cu²⁺, and Mn²⁺ ions. The enzyme activity was slightly inhibited by 0.5% Cd²⁺ ions. In contrast to Fe³⁺, the enzyme activity of pullulanase was only slightly reduced of Mn²⁺. Among the metal ions tested, Ca²⁺ had a very strong stimulating effect on the enzyme, increasing its activity.

Graph 8: Graph showing activity of enzyme with respect to different metal ions.
Metal ions are considered as interfering factors to enzyme activity so the various metal salts were tested in the present study. The calcium salt showed the minimum interference to activity as the media containing CaCl$_2$ as given the maximum enzyme activity.

### 3.5.8 Effect of polyols on the activity of pullulanase:

Effect of polyols on the activity of pullulanase from *Streptococcus* is shown in the graph 9. Activity of pullulanase at 50°C was significantly improved with the addition of sucrose into the reaction media. The highest improvement in stability (relative activity of 102.66) was obtained with sucrose. On the other hand, reduced activity of the enzyme was observed with the addition of other polyols. The enzyme was almost denatured with the presence of glycine.

Graph 9: Graph showing activity of enzyme with respect to different polyols.

Polyols are used as media supplement in very low quantity some time they enhance the production and promotes the secretion of enzymes in media. The sucrose was found as best promoter to enzyme activity.

### 3.5.9 Effect of various additives on the activity of pullulanase enzyme:

The effect of different additives on the production of pullulanase was studied using the basal culture medium supplemented with 2% of additives. The culture containing 2% of each additive at a time was incubated and tested for enzymatic activity. Among coconut water, vitamins, fruit juice, soya milks and were used 2% at a time. Coconut milk was best to enhance the pullulanase activity (98 IU). Graph 5.10 showing highest pullulanase activities as compare to other additives sources. The highest pullulanase activity may be due to inducible nature of maltose being hydrolytic product of starch.

Graph 10: Graph showing activity of enzyme with respect to different additives sources.

Different additive sources (vitamins, fruit juice, coconut milk, and soya milk) were examined in this study the coconut milk gave the best enzyme activity.

### 3.6 Partial purification of pullulanase:

The Pullulanase activity of crude enzyme was 67.66 IU. The 40 to 80% fraction of the crude enzyme under ammonium sulphate purification gave rise 98.0 IU and Gel filtration column chromatography of precipitated protein by ammonium sulphate gave rise 130.66 IU pullulanase activity which was greater than the 0-30% (98.0 IU) and 70-90% (130.66 IU). The enzyme purification by ammonium sulphate purification (2.02) and Gel filtration chromatography gave rise approximately (4.78) time purification (Table: 1). The termination of protein for calculation of specific activity was done by Biuret method, which is based on copper ions binding to peptide bonds of protein under alkaline conditions to give a violet or purple colour. The intensity of the charge transfer absorption bond resulting from the Cu-protein complex is linearly proportional to the amount of protein present in the solution.

Table 1: Partial purification of pullulanase from *Streptococci*.

| Stage                        | Volume (ml) | Total activity (units) | Total protein (mg) | Specific activity (U mg$^{-1}$) | Purification (fold) |
|------------------------------|-------------|------------------------|--------------------|-------------------------------|---------------------|
| Crude extract                | 100         | 67.66                  | 1.02               | 66.33                         | 1                   |
| 40,60 and 80% (NH$_4$)$_2$SO$_4$ ppt | 50          | 98.00                  | 0.62               | 156.8                         | 2.02                |
| Gel filtration column        | 25          | 130.66                 | 0.41               | 317.13                        | 4.78                |
| Chromatography               |             |                        |                    |                               |                     |
3.7 SDS-PAGE and molecular weight determination

![Figure 3: SDS PAGE profile for pullulanase](image)

Lane 1 molecular marker
Lane 2 purified pullulanase enzyme
Lane 3 contains whole cell lysate.

The SDS PAGE profile of purified pullulanase enzyme has shown in figure 3. In lane 2 purified proteins resolved between 100 and 75 KDa. This proves the presence of pullulanase enzyme in lane 2 as its molecular weight is 98 KDa.

4. Conclusion

The identification of enzyme producing strains was another important step for the completion of the project. In this present study the enzyme was isolated by the extremophilic strains of *Streptococci* isolated by some harsh conditions like high temperature, low moisture level and high pH. After the screening of extremophiles purification of enzyme was done with the help of column chromatography, molecular weight determination by SDS-PAGE. Pullulanase 98 KDa was successfully produced and purified by the isolated strains. As it is very stable at higher range of temperature and pH so can be used in many industrial processes. The media preparation is one of the most important objectives of this study has been achieved and the most appropriate combinations of media components can be stabilised with the help of this. In this process to study enzyme activity the enzyme substrate reaction was performed based on various parameters temperature 50°C, time 72 hours, and pH 6. As it is clearly stated after the detail analysis of all parameters that the pullulanase isolated in the present study is very stable and active at different parameters.

Various carbon sources were checked as media component and pullulan was observed with best enzyme activity, while maltose, sucrose and glucose were showing gradually decreasing but significant enzyme activity. Peptone given the maximum activity in nitrogen sources, the activity against mineral supplements potassium salt was observed maximum enzyme activity. The calcium salt showed the minimum interference to activity as the media containing CaCl$_2$ as given the maximum enzyme activity. In the Polyols the sucrose was found as best promoter to enzyme activity and different additive sources (vitamins, fruit juice, coconut milk, and soya milk) were examined in this study the coconut milk gave the best enzyme activity.

The Pullulanase activity of crude enzyme was 67.66 IU. The 80% fraction of the crude enzyme under ammonium sulphate purification gave rise 98.0 IU and Gel filtration column chromatography of precipitated protein by ammonium sulphate gave rise 130.66 IU pullulanase activity which was greater than the 0-30% (98.0 IU) and 70-90% (130.66 IU). And thus it can be used in various industrial processes where normal mesophilic enzymes cannot be applied.

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