A Novel Culture Medium Using Lettuce Plant (Lactuca Sativa) For Detection of Pullulanase Production by Paenibacillus Macerans Isolated from Agricultural Wastes

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Abstract. Pullulinase is an enzyme catalyzing the hydrolysis of pullulin forming maltotriose, panose and maltoligosaccharides that have a great prominence owing to their prospective use in medication, drink, diet and fine chemical industries. Because of the production of this enzyme is too expensive and characterized with low yield, there is a growing need to create a substrate to make the total process cheaper and more creative. In this study, the authors have reported pullulanase production from Paenibacillus macerans. Six isolates of Paenibacillus macerans were isolated from soil and agricultural wastes samples and screened for higher pullulanase production, Paenibacillus macerans isolated from second sample of wheat husk was gave superior inhibition zone around the colony as compared with other tested isolates. The activity of pullulanase was raised to 9.83U/ml when this strain was grown under the optimal conditions including using basal medium containing 1% (w/v) dried leaves of lettuce and 2% (w/v) peptone at pH 6, temperature of 50°C, inoculums size of 1.5 ml for 72 hour. The pullulanase exhibited highest activity (6.80U/ml) and (6.43U/ml) in presence of lettuce. Lettuce has aptitude as an efficient and economically suitable as compared with traditionally used substrates as pure pullulin and other types of saccharides.

Keywords: pullulanase, optimal conditions, screening, wheat, lettuce, yield.
1. Introduction

*Paenibacillus macerans* is a part of the Paenibacillaceae family which are facultative anaerobes [1]. *Paenibacillus macerans* defined as a nitrogen fixing and fermenting diazotroph bacteria exists in plants and soil but has also been located in blood cultures of infected infants [2]. Many species of *Paenibacillus* are authenticated to produce different enzymes involving in production of various chemicals [3]. Pullulan is a linear polymer of maltotriose, linked with glycosidic linkages α- (1→6) as well as used in various types of industries [4].

Pullulanases (E.C. 3.2.1.41, α -dextrin 6-glucano-hydrolase) are endo-acting debranching enzyme that particularly cleaves glycosidic linkages α (1,6) in pullulan related oligosaccharides as amyllopectin and starch [5]. Pullulanase classified as a type of amylase enzymes [6]. Its configuration is multi-domain protein possessing two or three catalytic residues [7].

Pullulanase is widely distributed in plants and bacteria [8]. It was first extracted by from *Klebsiella pneumonia* as reported by [2], pullulanase that produced from microorganisms attracts more concern due to its definite action on glycosidic linkages α-(1,6) in pullulan. There are many *Bacillus* spp. that produce pullulanases as *Bacillus deramificans*, *Bacillus cereus* & *Bacillus acidopullulyticus* [9]. Pullulanase authenticated with different applications like starch debranching [9]. The most distributed industrial application of this enzyme is the production of maltose or glucose syrups [10]. During starch processing industries, this enzyme is also used for resistant starch production, detergent, baking manufacturing and for cyclodextrins production. It can also be used as a control agent on dental plaque [11]. Enzymes derived from microbes work depends on the purpose of their application, concentration, temperature, pH and differentiated according to their chemical and physical factors [10]. Because of the expensive production process of pullulanase and low yield as well as for the strong productionin large-scale of this enzyme, there is a true need to use low cost substrates that are high in starch and to optimize its production conditions. For these reasons, the current study has aimed to determine the efficiency of using local plant derived substrate within the medium of pullulanase production by *Paenibacillus macerans* besides to determine the optimum conditions of medium for increasing the yield of this enzyme with lower cost.

2. Materials and methods

2.1. Samples Collection

The researchers have collected thirty samples from various sources in sterile containers; these samples were wheat husk, rice husk and soil.

2.2. Screening of pullulanase producers

After weighting one gram of each sample (rice husk, wheat husk and soil), these samples have serially diluted in sterile water and heated for 10 min in water bath at 80°C. A volume (0.1) ml from each diluted sample have spread on the surface of modified pullulan agar medium with ingredients displayed in (table 1)

| Ingredient | pullulan | NaCl | MgSO\(_4\).7H\(_2\)O | K\(_2\)HPO\(_4\) | KH\(_2\)PO\(_4\).7H\(_2\)O | Agar |
|------------|---------|------|-----------------|----------------|------------------------|-----|
| Amount g/l | (10)    | (2)  | (0.1)           | (0.17)         | (0.12)                 | 15  |
All these ingredients were prepared in g/l then pH of media have adjusted to 7.0. The petri dishes have then incubated at 30°C until the appearance of colonies according to [11]. pullulanase producers colonies was rounded with a clear hollow zone. The isolates were identified by microscopically, morphological characteristic in addition to gram stain and biochemical tests were used to identify Bacillus as reported [4].

2.3. Submerged fermentation for production of pullulanase

The selected Bacillus isolate have cultured in modified pullulan agar medium except that it does not contain agar. Then incubated for 2 days at 30°C. The culture was centrifuged for 10 min at 8000 rpm and the super layer was separated to use as (crude) enzyme.

2.4. Pullulanase activity

The activity was evaluated by determining the reduced sugar released from 3 ml of pullulan reaction mixture which consisting of the following ingredients:
1-A volume of (0.5 ml) pullulan (1% w/v)
2-A volume of (0.5 ml) of enzyme source (crude) in 2ml of sodium phosphate buffer (0.1 M, pH 6.5).
3-A volume of (0.5 ml) CaCl$_2$ (0.02% w/v).

After incubation for 20 min at 40°C, the tubes have been cooled in an ice bath to stop the reaction reducing sugar released by destroying of pullulan by enzyme was assayed through addition a volume of (1ml) of reagent (3, 5-dinitrosalicylic acid), then incubated the tubes in a water bath for (5 min.), and calculating the activity of enzyme at wave length 540nm [12]. One pullulanase unit defined as the quantity of enzyme, which released one micromole of reducing sugars as glucose per minute under standard assay conditions.

2.5. Optimization of culture circumstances for production of pullulanase

2.5.1. Influence of carbon sources:
A- The dried sweet potato, dried leaves of lettuce , grounded local red kidney beans, rice bran and corn bran were ground to obtain the powder. One percent (w/v) of each these agriculture wastes were added to production medium instead of pure pullulan.
B- The selected isolate has inoculated in liquid production medium with presence of different carbon sources (dried sweet potato, dried leaves of lettuce , grounded local red kidney beans, rice bran, corn bran, starch, glucose, sucrose) and incubated at 30°C for 48 hours at pH value 7. Then the pullulanase activity have been calculated as described above.

2.5.2. Influence of nitrogen sources:
A- Beef extract, peptone, tryptone, and yeast extract were used as nitrogen sources. One percent (w/v) of each these as nitrogen sources were added to production medium.
B- The selected isolate has inoculated in liquid production medium with presence of various nitrogen sources (beef extract, peptone, tryptone, and yeast extract) and incubated at 30°C for 48 hours at pH value 7. Then the pullulanase activity was calculated.

2.5.3. Influence of incubation temperature:
Selected isolate have inoculated in broth at various temperature values (37, 40, 45, 50, 55, 60)°C for 48 hours, then the activity was calculated.

2.5.4. Influence of pH value:
broth have prepared at different values of pH (4-9), the medium was inoculated and incubated at 50°C for hours, then the pullulanase activity was calculated.
2.5.5. Influence of incubation period:

Selected isolate have inoculated in liquid medium and incubated at 50°C for various times (1, 2, 3, 4, 5, and 6 days) and then the pullulanase activity was assayed.

2.5.6. Influence of inoculum volumes:

Different inoculum volumes of selected isolate have inoculated in broth (0.5, 1, 1.5, 2 and 2.5 ml) and incubated for 72 hours at 50°C, and then the pullulanase activity was calculated.

3. Results and discussion

3.1. Screening pullulanase producers

The bacterial isolates that isolated from soil and agricultural wastes exhibited a clear hollow zone around their colonies after incubation on pullulan agar plate and considered as producer for pullulanase as shown in figure (1). According to microscopic observation and biochemical tests, these six isolates were diagnosed as Paenibacillus macerans. The calculated ratio ranged between 0.5-4.3 (figure 2) and Paenibacillus macerans isolated from sample No.2 from wheat husk (W2) that isolated from wheat gave the maximum level for pullulanase production. The differences in the diameter of hydrolysis zone among Bacillus isolates return to diversities in genetic expression of pullulanase by Bacillus [9]. The later study elucidated that pullulanases are predominantly extracellular enzymes yielded by different bacterial genera, essentially by Bacillus sp.

![Figure 1: A clear hollow zone around Paenibacillus macerans isolate.](image-url)
3.2. Optimization of culture circumstances for pullulanase production

3.2.1. Influence of carbon source

The data obtained in this study established that among eight examined sources of carbon which used as a sole carbon and energy source the maximum pullulanase production yielded by using lettuce plant as a sole source for carbon since the pullulanase activity reached to (6.8U/ml) after incubation for 48 hour at 30°C. Therefore, lettuce considered the most excellent substrate for pullulanase production as compared with the other types of substrate as shown in (Figure 3). The source of carbon is the most significant raw material for microbial cultivation, since it has provide the essential material for energy production and microbial constituents required for cultivation [13]. In a study reported by [14] revealed that the utility of plant derived substrate for pullulanase production would not only help in the decrease of the production cost of the enzyme but will also help the environment by their elimination.

Figure 2. The ratio of pullulan hydrolysis zones by Paenibacillus macerans isolates from different sources; (S) Refer to soil, (R) refer to rice husk and (W) wheat husk.

Figure 3. Influence of carbon sources on pullulanase production by Paenibacillus macerans.
3.2.2. *Influence of nitrogen source*

Among the four N sources that used in current study, maximum pullulanase activity was recorded when the peptone was used as a sole nitrogen source (7.44 U/ml) as compared with other sources as displayed in (*figure 4*). In study by [15], authors explained the importance of nitrogen affected growth of bacteria by its crucial need to full the metabolic pathways. In addition, nitrogen improve and boosts growth of bacteria may be by increasing the production of essential enzymes. This result completely agreed with results showed by [9] which reported that pullulanase production by *Bacillus halodurans* and *Bacillus licheniformis* was stimulated by the addition of peptone.

![Figure 4: Influence of nitrogen sources on pullulanase production by *Paenibacillus macerans*.](image)

3.2.3. *Influence of incubation temperature*

The results displayed in (*figure 5*) demonstrated that highest production of pullulanase obtained after incubation at 50°C when the pullulanase activity reached (8.55 U/ml), while the maximum temperatures were not proper for production of pullulanase, the activity declined to 2.1 U/ml at 60°C. The temperature is important factor, which regulate enzyme production and stability, the excessive temperature affect the living cells metabolic processes through denaturation of enzymes [16]. The same result was obtained by [10] who showed that 50°C was the best temperature for pullulanase production by *Bacillus halodurans* and the optimum temperature was 40°C.
3.2. 4. Influence of pH value

Maximum pullulanase production yielded when the medium pH value adjusted to (6), at which the activity of the enzyme was reached to (8.98U/ml), while at pH higher or lower than 6 decreasing in enzyme activity was recorded as shown in (Figure 6).

As reported by [17]. The pH of the growth medium is one of the physical parameters that plays a crucial role in generating changes in the organism morphology and in secretion of enzyme also the previous study suggested that any alteration in pH value affect the structure of protein thus leading to decline in the activity of enzyme and may affect the interaction between enzyme and substrate, particularly when the active site has been distorted. Since study of [18] revealed that the optimum pullanase production by soil bacteria at pH ranged (6-7) and the best production of pullulanase by Bacillus halodurans was obtained at pH value (10.0).

Figure 5: Influence of temperatures on pullulanase production by Paenibacillus macerans.

Figure 6. Influence of pH values on pullulanase production by Paenibacillus macerans.
3.2.5. Influence of incubation period
The results showed that after 1 day of incubation the production of enzyme was initiated with regular growing in productivity with rising in incubation period as displayed in (figure 7). The activity value of pullulanase reached after 3 days of incubation to maximum level (9.76U/ml), followed by falling off in enzyme activity after this period. [19] Explained this result. This decline in enzymatic activity may due to denaturation process or degradation of the substrate. This resulted by interaction between substrate with other ingredients in the medium. High level of pullulanase production by Bacillus cereus could be obtained after 48h hour incubation period [11].

Figure 7. Influence of incubation periods on pullulanase production by Paenibacillus macerans.

3.2.6. Influence of inoculum volume
The findings showed activity of pullulanase slightly raised with the increase of the inoculum size until 1.5 ml with pullulanase activity of (9.83U/ml), and then decreased with increasing in inoculum size as illustrated in (figure 8). At lower inoculum level, the lowest enzyme production might be resulted from less viable cells number that participating in the production and require more time to grow to reach to optimum number and produce the enzyme by utilizing the substrate in medium [4]. In contrast, higher inoculum size leads to low level of enzyme production , this might be as a result of either decrease in availability of essential nutrient for the large number of viable cells, or quick accumulation of toxic byproducts [20]. As reported by [21],The inoculum amount affects the adaptation phase because many species of bacteria organize gene expression by quorum sensing so that the suitable inoculum amount is necessary for bacterial individuals to achieve communication with each other using signaling molecules stimulate bacterial growth.
4. Conclusions
An advantage of plant derived substrate (pullulan) for pullulanase production would not only help in the decrease of the production cost of pullulanase but will also help the environment by their elimination the accumulation of agricultural wastes. Lettuce has demonstrated as an efficient and much economically suitable as compared with traditionally used substrates as pure pullulin to make the total process much cheaper and more productivity.

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