

Production optimization of pullulanase enzyme produced by *Bacillus cereus* isolated from Syrian sources

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Abstract

The objective of this study was to isolate, identification the highest pullulanase that produced by Syrian bacteria from different environmental sources such as baking wastes, soil, and food wastes samples, then optimization production of pullulanase. 17 isolates was positive for pullulanase production, The isolate P8 demonstrated the highest extracellular pullulanase activity, it was identified as *Bacillus cereus* based on Morphological and biochemical experiments. Culture conditions parameters for production enzyme were optimized partial characterization of extracellular pullulanase from *Bacillus cereus*. The results showed maximum pullulanase production occurred after 48 h, 10⁷ colony frame unit (cfu), at pH 7.5. The optimum temperature for pullulanase production was found to be 37°C in the presence of 1% soluble pullulan as a carbon source and 0.5% tryptone. The activity of pullulanase at this optimal conditions was (1.18 U/ml) which is 2.14 fold higher than before optimization. By characterization of pullulanase, highest activity observed at pH 6.0 and 50°C.

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Keywords

Pullulanase

Bacillus

Pullulan

Optimization

Introduction

In the recent years the world tends toward using renewable, cheap and readily available materials such as the biomass to produce or convert these materials into useful substances. Utilize the microorganisms by their enzymes are widely used to convert the biomass into target products (Sweeney and Xu, 2012). However many of these processes require high temperature therefore enzymes which are operationally stable at high temperature is essential to complete the task (Vieille and Zeikus, 2001). Thermostable enzymes can be used in different application such as technical enzymes, food enzymes, and animal feed enzymes (Adrio and Demain, 2014). The largest section is technical enzymes where enzymes used for detergents and pulp and paper processing (Li *et al.*, 2012).

One of the candidate of the industrial enzymes is Pullulanase; an important debranching enzyme that has been widely used to hydrolyse the α -1,6 glucosidic in starch, amylopectin, pullulan, and related oligosaccharides (Hii *et al.*, 2012). Several amylolytic enzymes, such as α -amylase, β -amylase and glucoamylase, with different specificities can contribute to starch degradation (Martin and štefan, 2007). These enzymes, all of which hydrolyze α -1,4-glucosidic bonds, are capable of amylose degradation, yielding glucose, maltose, maltotriose, and other oligosaccharides. However, in the absence

of a “debranching” enzyme capable of hydrolyzing α -1,6-glucosidic bonds, amylopectin degradation is incomplete (Ryan *et al.*, 2006). In combination with other amylolytic enzymes, pullulanase enables a complete and efficient conversion of the branched polysaccharides into small fermentable sugars such as fructose syrup and maltose during saccharification process (Norman, 1982), and enhances the reaction rate thus it speeds up the production of sugars resulting in cost reduction of producing sugars from starch (Douglas Crabb and Mitchinson, 1997). The other application of pullulanases is the detergent industry (Adrio and Demain, 2014), dental plaque control agent (Marotta *et al.*, 2002), enhancement of cyclodextrins production (Nishank Prakash *et al.*, 2012). Commonly cyclodextrin glycosyltransferase (CGTase) is used with α -amylase. But the α -1,6-glycosidic bond of the amylopectin blocks the action of the CGTase (Szejtli, 1998b). These bonds are broken by the pullulanase enzyme hence increasing the percentage yield of the cyclodextrin production (Szejtli, 1998a). Microbial Pullulanase is desired in the industry due to its specific action on α -1,6 linkages in pullulan, a linear α -glucan consisting essentially of maltotriosyl units connected by 1,6- α -bonds. In addition, pullulanases hydrolyse the α -1,6 glucosidic bond in pullulan and amylopectin, while isoamylase can only hydrolyse the α -1,6 bond in amylopectin and glycogen.

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Pullulanase is produced by various microorganisms such as *B. acidopullulyticus*, *Klebsiella planticola*, *B. deramificans*, *B. cereus* FDA-13, and *Geobacillus stearothermophilus* (Hii *et al.*, 2012). The development of fermentation processes during the later part of the last century, aimed specifically at the production of enzymes by use of selected production strains, made it possible to manufacture enzymes as purified, well-characterized preparations even on a large scale. This development allowed the introduction of enzymes into true industrial products and processes (Kirk *et al.*, 2002). Medium development can be used to identify the ideal growth and production environment of a cell (Abu Samah *et al.*, 2008).

Materials and Method

Samples collection and isolation of bacterial cultures

Soil samples from 5 cm depth, baking wastes and food wastes samples were collected from different location in Syria. Stock samples were stored at 4°C. Isolation of bacteria was performed by preparing five serial dilution of 1 gram of the processed sample and then spread to Nutrient agar plates. The plates were incubated at 37°C for 24 h. The bacterial isolates were further subcultured on the respective mediums in order to obtain pure culture.

Primary screening of pullulanase producing bacteria

The isolates were screened by cultivate it in nutrient broth at 37°C for 24 hr. and plating 5 µl from each on pullulan agar medium (g/l): pullulan (10), NaCl (2), MgSO₄.7H₂O (0.1), K₂HPO₄ (0.17), KH₂PO₄.7H₂O (0.12) and agar (15), pH 7.5 and then the plates were incubated at 37°C for 48 h. Presence of transparent zone of hydrolysis around the colonies after 48 h of incubation indicates presence of pullulanase. The halo zone diameter (H) in mm and colony diameter (C) in mm were measured. Degradation efficiency of all the pullulanase producers isolates was calculated using the formula (H-C)/C (%) (Sreedevi and Reddy, 2013). The bacterial isolates which showed efficiency above 50 % were selected and subjected to secondary screening.

Secondary screening of pullulanase producing bacteria

Freshly isolates that have degradation efficiency above 50% were prepared to inoculate the production medium. For the preparation of inoculum a loop full of bacterial isolates was transferred in 50 ml of pullulan medium without agar. The flask was loaded on a rotary shaker incubator at a speed of 200 rpm

at 37°C for 24 h. 100 ml of the production medium (same as inoculation medium) was inoculated with 107 cfu of bacterial inoculum. The flask was loaded on a rotary shaker incubator at a speed of 200 rpm at 37°C for 48 h. After incubation, fermented broth was centrifuged at 7000 rpm for 10 min in a cooling centrifuge. Supernatant was collected and used for the pullulanase assay.

Identification of the isolate

The selected bacterial isolate that show high pullulanase activity was identified based on the morphological and biochemical parameters as described in the Bergey's manual of systematic bacteriology (Bergey, 2000).

Enzyme assay for pullulanase enzyme

Pullulanase activity was assayed by measuring the amount of reducing sugars liberated during the action of the enzyme on a pullulan substrate. Pullulanase activity was measured in a reaction mixture consisted of 0.46 mL of 1% pullulan in 0.05 M potassium phosphate buffer, pH 7, and an appropriate concentration of the extracellular enzyme crude (0.04 mL), incubated at 40°C for 10 min. After incubation the reaction was stopped by immediate cooling in an ice bath and by addition of the dinitrosalicylic acid (DNS) reagent (Miller, 1959). A unit of pullulanase activity was defined as the amount of pullulanase required to catalyze the liberation of reducing sugar equivalent to one µmol of D-glucose per minute under the assay conditions (Ara *et al.*, 1995).

Effect of incubation period, growth and inoculum concentration

The effect of incubation period and growth isolate on the enzyme production was tested by incubate basal media for 7 days. The fermentation media were sampled and tested every day for pullulanase production. The growth isolate was tested by observation of optical density on 600 nm. The effect of inoculum concentration was tested by cultivating *B. cereus* in the basal media at different range of inoculum concentration, 10⁴, 10⁵, 10⁶, 10⁷, 10⁸, 10⁹ cfu. The fermentation media were tested after 48 h for pullulanase production.

Effect of medium ph and temperature incubation

The influence of initial medium pH on pullulanase production was assessed by cultivating *B. cereus* in the basal media of pH ranging from 5.5 to 9.5 The effect of temperature was studied by performing the fermentation at different range of temperatures, 25, 30, 35, 37, 40, 45 and 50°C. The fermentation media

were tested post 48 h for pullulanase production.

Effect of various carbon and organic nitrogen sources

To determine the effect of different carbon sources for enzyme production, *Bacillus cereus* was inoculated into 100 ml serum vials containing basal media supplemented with: 1% w/v of individual carbon sources viz. Pullulan, starch, maltose and corn flour for study of effect carbon sources. The effect of different nitrogen sources was tested by adding 0.5% w/v of individual nitrogen source viz. casein, tryptone, peptone and yeast extract. All of this media were sterilized separately at 121°C for 1 h. The vials were incubated at 37°C for 48 h. The amount of enzyme produced was estimated under standard assay conditions described above.

Effect of pH on pullulanase activity

The optimal pH for enzyme activity was determined by changing the pH of the assay reaction mixture using buffers of (0.1 M): sodium acetate (pH 5.5), potassium phosphate (pH 6.0–7.0), Tris-HCl (pH 8), glycine-NaOH buffer (pH 9) and 1% soluble pullulan as substrate.

Effect of temperature on pullulanase activity and stability

The optimum temperature for the enzyme activity was evaluated by measuring the pullulanase activity at different temperatures (25–65°C) in 0.05 M potassium phosphate buffer (pH 7.5) and 1% soluble pullulan. The temperature stability was determined by measuring the residual activity at 50°C, after incubation of the enzyme in different temperature ranging from 30 to 70°C for 60 min at optimum pH. All the experiments were conducted in triplicate.

Statistical analysis

All the experiments were conducted in triplicate. Statistical difference was analyzed for experiments of optimization by one way ANOVA test using SPSS 13.0. $P < 0.05$ was considered as significant at the 0.05 level of confidence. Y bars indicate the standard deviation of mean value.

Results

Isolation, screening and identification of pullulytic bacteria

Fifty bacterial isolates were obtained from three different environmental sources including baking wastes, soil, and food wastes. The primary screening of these bacterial isolates on pullulan agar showed that 17 isolates have pullulytic ability by forming zone

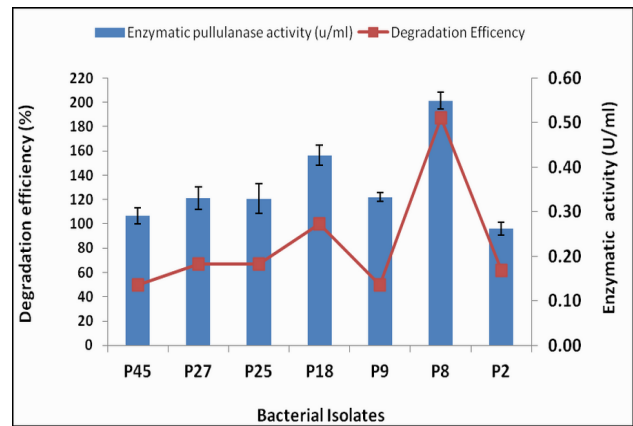


Figure 1. Extracellular Pullulanase production in broth medium from selected bacterial isolates obtained from different sources and its efficiency on solid medium. pullulanase production by P8 isolate showed significant ($P < 0.05$) with others.

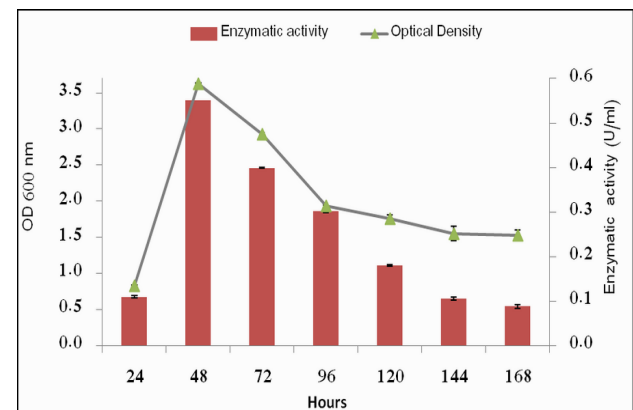


Figure 2. Effect of incubation period on pullulanase production by *B. cereus*. pullulanase production showed significant ($P < 0.05$) after incubation for 48 h compared with others.

of transparent, and 7 isolates showed degradation efficiency above 50%. These 7 isolates were subjected to secondary screening for pullulanase production using submerged fermentation. The culture filtrate of isolate P8 showed initial maximum pullulanase activity (0.549 U/ml) and 187.5 % as maximum value to degradation, this result was considered as control before optimization. P8 was selected for further studies (Figure 1). The morphological and biochemical characteristics, isolate P8 was identified as *B. cereus*.

As shown in (Figure 2) that pullulanase production and growth clearly started increasing after incubation of medium for 24 h and both were maximum after incubation of medium for 48 h where the extracellular pullulanase production has increased from 0.11 U/ml (at 24 h) to 0.55 U/ml (at 48 h) with concomitant decrease in pullulanase production after 48 h.

Pullulanase production was gradually increased as the inoculum concentration increases from 10^4 ,

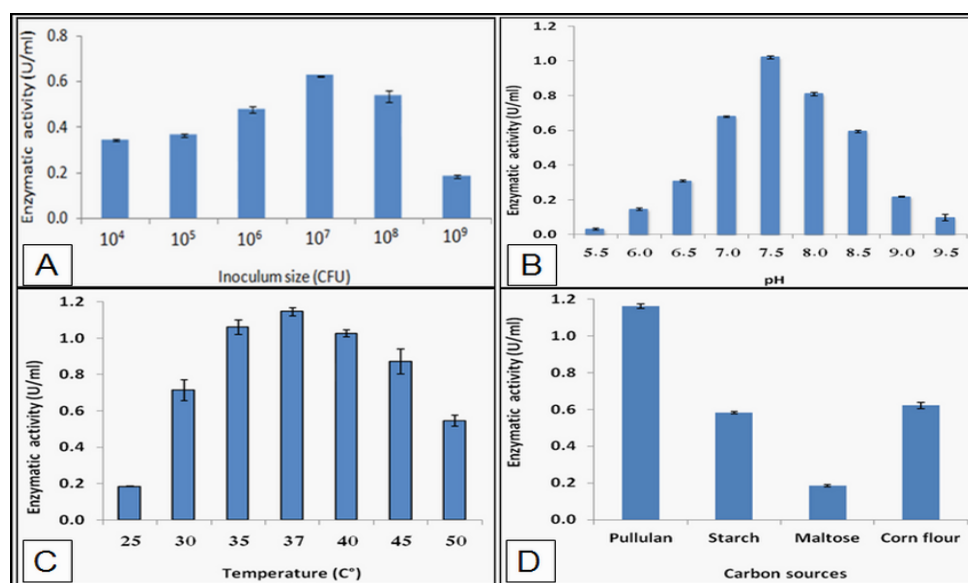


Figure 3. Effect of: Inoculum concentration (A), Media pH (B), Temperature incubation (C), Carbon sources on pullulanase Production from *B. cereus*. pullulanase production showed significant ($P < 0.05$) at 10^7 cfu, pH 7.5, 37°C and using pullulan by compared with specific factors to each experiment each separately

10^5 , 10^6 , 10^7 cfu with maximum pullulanase at 10^7 cfu (0.62 U/ml), and was gradually decreased at increasing the inoculum concentration from 10^8 , 10^9 cfu (Figure 3 A).

The production of pullulanase increased as pH of the medium increases, there was good pullulanase production at pH values 7, 7.5, 8, 8.5 with maximum pullulanase production was achieved at pH 7.5 (1.02 U/ml) as shown in (Figure 3B). After pH 7.5 there was a decrease in enzyme production. *B. cereus* was capable of producing pullulanase in the temperature incubation range of 25- 50°C of production medium with good production between 30°C - 45°C and maximum production at 37°C (1.14 U/ml) (Figure 3C).

Among the different carbon and nitrogen sources tested, pullulan was more preferred for extracellular pullulanase production. As shown in (Figure 3C), Pullulan was the best carbon for pullulanase production (1.16 U/ml) comparative with others substrate, and there wasn't difference between starch or corn flour for pullulanase production (0.78, 0.62 U/ml) respectively, but maltose was the least effect (0.19 U/ml).

There were no difference for pullulanase production between tryptone and peptone (1.18, 1.17 U/ml) respectively, which were the best organic nitrogen source comparative with yeast extract and casein (0.88, 0.55 U/ml) respectively (Figure 4A).

A pH range from 4 to 7.5 was used to study the effect of pH on pullulanase activity and the result given in (Figure 4B). A relatively low level of activity was observed at pH values 4. Pullulanase activity of

Bacillus cereus reached its maximum at pH 6. At pH 6.5 and 7, Pullulanase retained 92.1 % and 69 % from its activity, respectively. The results illustrated in (Figure 4C) indicate that the optimum temperature for pullulanase activity was 50°C , maintained about 62.2 % of the maximal enzyme activity at 60°C , and maintained about 72 % of the maximal enzyme activity at 35°C .

Thermal stability studies were carried out by preincubation the enzyme up to 1 h in the range of temperatures from 30 – 70°C (Figure. 4 D). The pullulanase remained stable after 1 h of incubation at 30°C , and at 40°C the residual pullulanase activity was 79.21%. At 50°C , the enzyme retains 67.13% activity after 1 h, while at 60°C and 70°C the residual pullulanase activity was 30.53% and 7.32% respectively.

Discussion

Present study shows that P8 isolate was the best from others in primary and secondary screening for production of extracellular pullulanase enzyme where showed maximum pullulanase activity (0.49 U/ml). Using morphological and biochemical characteristics, the selected isolate P8 was identified as *B. cereus*, this results confirm that the genus *Bacillus* produces a large variety of extracellular enzymes, of which amylases are of particularly considerable industrial importance (Deb *et al.*, 2013), and are in agreement with the earlier reports that pullulanases are predominantly extracellular enzymes produced by a variety of bacteria, mainly by *B. cereus* (Bakshi

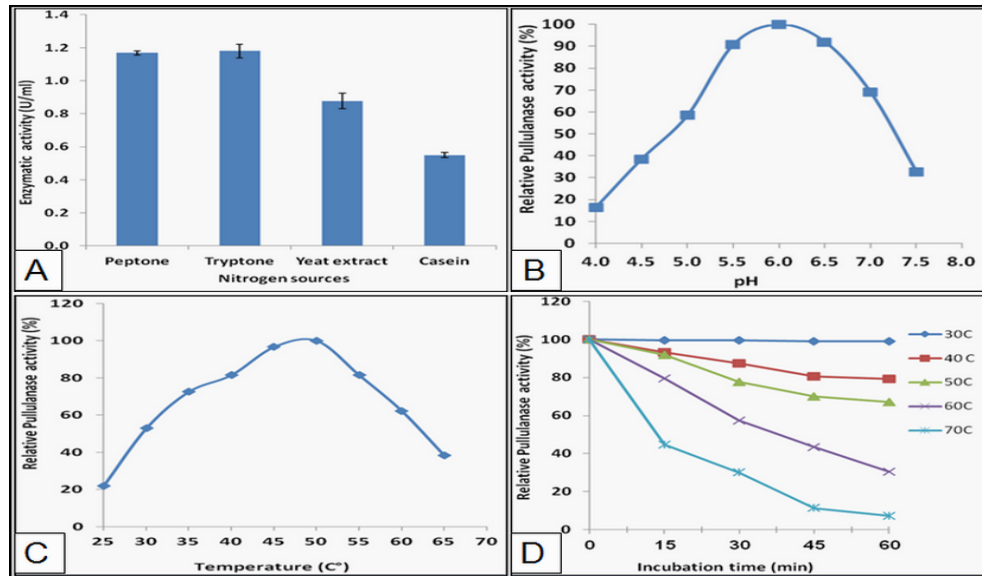


Figure 4. Effect of different organic nitrogen source (A) on pullulanase production by *B. cereus*, pullulanase production showed significant ($P < 0.05$) using tryptone compared with yeast extract and casein, while no significant ($P > 0.05$) compared with peptone, Influence of pH (B), temperature (C) on the activity of pullulanase produced from *B. cereus*. Relative activity was determined at pH 6, and 50°C, Thermostability of pullulanase from *B. cereus* (D). Activity of pullulanase showed significant ($P < 0.05$) at an optimal conditions compared with specific factors to each experiment each separately.

et al., 1992), *Bacillus* sp. (Teague *et al.*, 1992; Uhlig and Linsmaier-Bednar, 1998; Nair *et al.*, 2007).

Maximum growth and enzyme production was reached in 48 h of incubation, further incubation up to 48 h decreased growth and enzyme production. However, With the increase in incubation time (48 h), Similar results were reported from *Bacillus* sp. in the production of pullulanase (Khalaf and Aldeen, 2013), other worker showed a high level of pullulanase production by *Bacillus halodurans* was achieved after 5 days incubation period (Asha *et al.*, 2013), difference these results in pullulanase yields from others depend on strain, medium composition and culture conditions. Maximum pullulanase production was achieved at 10^7 cfu, less enzyme production at lower inoculum level ($<10^7$ cfu) might be because less number of viable cells in the production medium require more time to grow to an optimum number to utilize the nutrients in substrate and for enzyme production (Wind *et al.*, 1994). However, less enzyme production at higher inoculum level ($>10^7$ cfu) may be due to decreased nutrient availability for the large number of viable cells, or rapid accumulation of toxic metabolites (Asoodeh and Lagzian, 2012). The pH values of the culture strongly affects many enzymatic processes and transport of compounds across the cell membrane. Maximum pullulanase production was achieved at pH 7.5 by *B. cereus*. The production of pullulanase increased as pH of the medium increases and reaches maximum (1.02

U/ml) at pH 7.5. The obtained results coincide with Mrudula (2011) who has reported that pullulanase production was maximum at pH 7.5 for *Clostridium thermosulfurogenes* SVM17. Similarly Borriss (1987) reported maximum pullulanase production at pH 9-13. Increase in temperature beyond 37°C led to decline in production of enzyme proving that temperature plays a major role in pullulanase production. These results are in accordance with Asha (2013) who reported that pullulanase production was capable by *B. halodurans* in the range of 28–65°C with maximum production at 37°C. The addition of carbon source in the form of either monosaccharides or polysaccharides could influence the production of enzyme (Saxena *et al.*, 2007). Among the carbon sources, pullulan and starch were found to support pullulanase production. *B. cereus* showed high enzyme yield (1.16 U/ml), when pullulan was used as carbon source. Similar observation was noticed for an enhancement of pullulanase synthesis by pullulan in *B. cereus* FDTA-13 (Nair *et al.*, 2007) and in *B. halodurans* was also reported with (Asha *et al.*, 2013). Among the nitrogen sources, tryptone and peptone produced maximum pullulanase (1.18 U/ml), Similar observation was noticed for *B. halodurans* (Asha *et al.*, 2013) and *Clostridium thermosulfurogenes* SV9 (Swamy and Seenayya, 1996).

Enzyme activity is markedly affected by pH. This is because substrate binding and catalysis are often dependent on charge distribution on both, substrate

and, in particular enzyme molecules (Shah and Madamwar, 2005). Pullulanase activity of *B. cereus* reached its maximum at pH 6. This indicates that pullulanase activity from *B. cereus* has high activity at neutral pH. Similar results were observed for *Bacillus* sp (Brunswick *et al.*, 1999; Ben Messaoud *et al.*, 2002). pullulanase activity indicates that the optimum temperature for pullulanase activity was 50°C. similar results were observed *B. halodurans*, *Bacillus* sp. KSM-137 was achieved at 50°C (Ara *et al.*, 1995; Asha *et al.*, 2013). On the other hand, *Bacillus* sp. US149 (Roy *et al.*, 2003), *B. acidopullulyticus* (Stefanova *et al.*, 1999) presented optimal temperature for a maximum pullulanase activity at 60°C. Thermal stability studies indicates that the suitable temperature range for industrial application for pullulanase from *B. cereus* was 30-50°C.

Conclusions

The current results of this research demonstrate effect of culture conditions on *B. cereus* to increase producing of pullulanase enzyme. *B. cereus* can be a potential producer of extracellular pullulanase for biotechnological applications.

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