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Isolation and Identification of a New *Bacillus cereus* Strain and Characterization of its Neopullulanase

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Abstract

Identification and use of more efficient enzymes in the food and pharmaceutical industries is the focus of many researchers. The aim of this study was to search for a new bacterial strain capable of producing high levels of pullulanase applicable to biotechnology, the starch bioprocessing and food industries. A new pullulan hydrolyzing *Bacillus* strain was isolated and designated SDK2. Morphological and biochemical tests identified the strain as a putative *Bacillus cereus* strain, which was further characterized and confirmed through 16s rRNA sequencing, and was submitted to GeneBank, under the accession number FR6864500. Quantitative analysis of the strain's pullulanase activity was carried out by the Dintrosalicyclic (DNS) acid-based assay. Thin layer chromatography (TLC) of the culture supernatant, identified the extracellular pullulanase as neopullulanase. Effects of temperature and pH on pullulanase activity were also studied. The optimum conditions for enzyme activity, as represented by 60°C and a pH of 7, resulted in an activity of 13.43 U/ml, which is much higher than some of the previously reported activities. However, growth of *B. cereus* SDK2 was also observed at a pH range of 5 to 10, and temperatures of 30°C to 50°C. The effect of metal ions and reagents, such as Mg^{+2}, Ca^{+2}, Zn^{+2}, Cu^{+2}, Fe^{+2}, Ni^{+2} on enzyme activity showed that Ca^{+2} ions increased pullulan activity, whereas the other ions and reagents inhibited pullulanase activity. The ability of *B. cereus* SDK2 to produce high levels of neopullulanase stable at 60°C that can generate panose from pullulan, make this newly isolated strain a valuable source of debranching enzyme for biotechnology, the starch bioprocess and medical industries.

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1. Introduction

Starch, as the most common storage polysaccharide in plants, is an inexpensive substrate with extensive cost-effective benefits for the food industry [1]. Starch is made up of two components; the linear polymer known as amylose and the branched amylopectin. The entire carbohydrate molecule contains α-glucose units that are linked together with α-1, 4 and α-1, 6 glycosidic linkages [2, 3]. The α-amylase family consists of several enzymes that convert starch and related substrates to mono- and oligosaccharides, which includes α-amylase, pullulanase, cyclomaltodextrinases and cyclodextrin glycosyltransferase. Pullulanase (pullulan-6-glucanohydrolase) is a debranching enzyme that specifically acts on the α-1,6 linkages in pullulan, starch and related polysaccharides. This enzyme, as a starch debranching enzyme, is especially important in biotechnology and the starch bioprocessing industry and is used with other enzymes, such as α-amylase, β-amylase and glucoamylase for improvement of the saccharification rate and yield to produce sugar and

glucose syrups [4, 5, 6]. There are numerous reports regarding mesophilic, thermophilic and hyper-thermophilic bacteria and archaea that produce pullulanases. In fact, industrial processes mostly prefer the extracellular enzymes that are secreted by microorganisms. So searching for new bacteria that produce more and efficient pullulanases has always been an ongoing process [7, 8]. Pullulan is a linear polymer that is composed of maltotriose units linked by α-1,6 glycosidic linkages. Based on the substrate specificity and the type of end products, pullulan-hydrolyzing enzymes have been identified and classified into 5 groups [9], which include (i) pullulanase type I (EC 3.2.1.41) that attacks α-1,6 glycosidic linkages of pullulan (or the branched substrate) and produces maltotriose; (ii) pullulanase type II (amylopullulanase) is able to attack α-1,4 glycosidic linkages of pullulan (and related polysaccharides) despite the presence of α-1,6 glycosidic linkages, producing maltotriose, maltose and glucose; (iii) pullulan hydrolase type I (neopullulanase, EC 3.2.1.135) is able to attack the α-1,4 glycosidic linkages of pullulan, producing panose; (IV) pullulan hydrolase type II (isopullulanase, EC 3.2.1.57) acts on pullulan producing isopanose; (V) and pullulan hydrolase type III is able to attack both α-1,4 and α-1,6 glycosidic linkages of pullulan, producing maltotriose, maltose and panose [9]. All pullulanases, with the exception of neopullulanase and isopullulanase, are unable to act on cyclodextrins. Such kinds of enzymes are known as cyclodextrinases that are capable of acting on cyclodextrins much faster than starch [10]. Pullulanases are used at or above 60°C for the saccharification process, so thermal stability of these enzymes is of vital importance to such industries [7]. In accordance with the significant role of this enzyme in Biotechnology and the starch industry and the saccharification process, this study aimed to search for a new bacterial strain capable of producing high levels of pullulanase applicable to such industries.

2. Materials and Methods

2.1. Bacterial strains and culture

Bacterial strains were isolated from various locations of three industrial sites that included non-alcoholic beverage, gluten and flour companies, in Tehran, Iran. Samples taken from these sites were serially diluted with distilled water and spread onto Luria bertani (LB) agar plates, and then incubated for 24 h at 37°C. Individual colonies were then picked resulting in the selection of 73 amylolytic isolates. The 73 samples were subsequently cultured on pullulan agar (gl^{-1}; 2, pullulan; 16, agar) and incubated at 37°C for 48h. Pullulan-degrading bacteria were identified by the presence of clear zones (haloes) around colonies, after immersing the culture in ethanol (99 % (v/v)) for 3 h [11]. Consequently, colonies with the larger and more transparent (stronger) haloes were selected, from which the best strain was selected for identification and further analysis.

2.2. Bacterial identification and sequencing

Identification was carried out using morphological (Gram staining and light microscopy), biochemical (API kit (Biomerieux Co., France) and molecular methods (16sS rRNA sequencing). For the purpose of molecular identification, genomic DNA was extracted using a DNA extraction kit (Roche Co., Germany) and purified according to Sambrook and Russsell [12]. The purity of the DNA was checked spectrophotometrically by measuring A260/A280.

The PCR amplification of the 16S rRNA gene from the template (extracted genomic DNA of the putative *Bacillus cereus*, designated SDK2) was carried out with two universal primers; forward (5'-AGAGTTTGATCCTGGCTTA-3') and reverse (5'-TAAGGAGGTGATCCAGC-3'). PCR was carried out in a thermocycler with the following program: (1) an initial denaturation at 95°C for 10 min, (followed by 30 cycles each consisting of 1 min at 94°C, 40s at 54°C and 1 min at 72°C, with a final extension at 72°C for 10 min [12]. The amplified products were purified with the High Pure PCR Product purification kit (Roch Co, Germany) and DNA sequencing was then carried out on both strands. The Blast search algorithm (http://www.ebi.ac.uk/embl/) was used for alignment and homology search. The 16s rRNA sequence of the identified bacterial strain was submitted to Genbank under the accession no. FR686500.

2.3. Growth conditions for pullulan production

Modified BSS (*Bacillus* Spizizen Salts) minimal medium was selected as the cultivation medium that had the following composition (gl^{-1}); 14, K_2HPO_4; 6, KH_2PO_4; 2, $(NH_4)_2SO_4$; 0.2, Trisodium citrate; 0.2, $MgSO_4.2H_2O$; and trace elements solution ($MgCl_2$, $ZnSO_4$, $FeCl_3$, $CaCl_2$, at the final concentration of 10^{-6}M). The pH of the BSS medium was adjusted to 7. *Bacillus cereus* SDK2 was grown LB medium for 18 h at 37°C, with shaking at 200 rpm, and was then used to inoculate BSS medium (at a concentration of 1% (v/v)) containing 6 gl^{-1} of pullulan (% 0.6 (v/v)), and incubated at 37°C for 48 h at 200 rpm [7, 8, 9].

Sampling from the culture medium was carried out every 3 h and growth was monitored by measuring the optical density of the culture medium at 600 nm using a UV/VIS spectrophotometer (Beckman Co, Germany) [13]. Samples collected at regular time intervals were then centrifuged at 5000 g for 15 min at 4°C. The resulting cell-free supernatant was used as a crude source of enzyme in the following enzyme assays.

2.4. Enzyme assay

Pullulanase activity was determined by modification of the method by Kanno and Tomimura

(1985), at 37°C as the amount of enzyme that is required to produce reduced sugars from pullulan [14]. A 150 µl sample of the cell- free supernatant was added to 150 µl of 0.1M sodium phosphate buffer pH 7 containing 1% (w/v) pullulan. A same volume of cell-free supernatant in 0.1M sodium phosphate buffer (pH 7) devoid of pullulan was used as a blank. The resulting mixtures were then incubated at 37°C for 1 h, and the reaction was stopped by transferring the samples to ice. The released sugars were assayed by the DNS method. Once the DNS reagent (Sigma Co, Germany) was added, the resulting mixtures were heated at 100°C for 5 min. The reactions were stopped again by transferring samples to ice. The reducing sugars were measured using glucose as standard at 550nm [15, 16]. One unit of pullulanase was defined as the amount of enzyme required to produce reducing sugars equivalent to one µM of glucose produced per min under assay conditions, and expressed as U/ml [8].

2.5. Identification of pullulan hydrolysis products

In order to identify the products of pullulan hydrolysis, thin layer chromatography (TLC) using silica gel sheets 60 F254 (Merck Co, Germany) was employed. A modification of the method by Lin and Leu [17] was applied, where a butanol: ethanol: water (3:1:1[v/v/v]) mixture was used as the eluent [17]. A mixture containing glucose, maltose, maltotriose, maltotetraose, maltopentaose and panose (Sigma Co, Germany) were used as the standard. Culture supernatants were incubated with 1% (w/v) pullulan substrate at 60°C for 1 h and the resulting products of this reaction were spot inoculated onto TLC sheets.

2.6. Effect of temperature and pH on enzyme activity

The optimum temperature and pH for maximum enzyme activity was measured by incubating the culture supernatant containing the extracellular enzyme at different temperatures and different pH values, in 0.1mM sodium phosphate buffer for 1 h [10, 18]. The resulting enzyme activity was measured as described previously.

2.7. Effect of metal ions and other reagents on enzyme activity

The effect of metal ions, such as Mg^{+2}, Ca^{+2}, Zn^{+2}, Cu^{+2}, Fe^{+2}, Ni^{+2}on the crude enzyme samples were studied at two different concentrations of 2 mM and 0.2 mM [10]. The culture supernatants were incubated with metal ions in 0.1mM sodium phosphate buffer (pH 7) at 60°C for 60 min, and the resulting activity was measured as described above [18].

The effect of other reagents, such as ammonium sulfate, EDTA, SDS and urea at different concentrations, on enzyme activity were also studied as described above [10]. Reaction mixture devoid of metal ions and reagents was used as a control.

2.8. Statistical Analysis

Statistical analyses were carried out using the SPSS software version 16.0 (SPSS, Chicago, IL, USA). The independent Student's t-test was used to analyze significant differences. All experiments were replicated three times. Data throughout this study (Figures 1, 2 and 3) represent the mean ± standard deviation (SD) for three independent experiments (n=3), and error bars represent the SD. In all cases the results were statistically significant when p-values reported less than 0.05 ($P<0.05$).

3. Results and Discussion

In this study, 73 native amylolytic isolates obtained from three industrial sites in Iran were screened for the ability to produce high levels of pullulanase. The strain with the largest and strongest halo on pullulan agar had the highest pullulanase activity of 13.43 U/ml (Figure 1). Morphological identification of this isolate showed chain-forming, Gram-positive rods with sub-terminal spores. Biochemical tests using the API kit indicated a strain very similar to *Bacillus cereus*. Subsequent sequencing of the SDK2 16S rRNA gene confirmed the results of morphological and biochemical methods. Homology search via BLAST search and other bioinformatics methods, such as Chromas (2.33 version) and Bioedit (7.0.5 version) showed 99% similarity with *Bacillus cereus*. Hence, the SDK2 isolate was identified as *B. cereus* (designated SDK2), and its 16S rRNA gene sequence was submitted to Genbank under accession number FR686500.

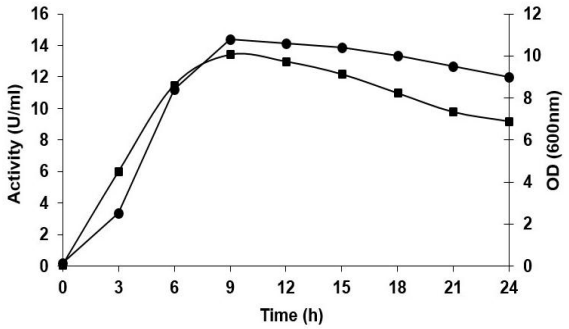

Figure 1. Growth and pullulanase activity of *B. cereus* SDK2 in BSS medium containing 0.6 % (v/v) pullulan, at 37°C. Symbols: ●, Cell growth; ■, pullulanase activity. Data in this study represent the mean ± SD for three independent experiments. P-value of <0.05 was considered significant.

Cultivation of *B. cereus* SDK2 in the presence of pullulan under different conditions and its extracellular enzyme activity were also studied. The isolate was able to grow well at a wide range of pH values (5-9) and temperatures (30-50°C), with optimal growth occurring at a pH value of 7 and a temperature of 37°C (data not shown). A notable observation was that maximum pullulanase production by this strain (13.43 U/ml) occurred at the end of the logarithmic

phase of growth (Figure 1) and this happened not only for this strain but also in each of the other isolated strains that had pullulanase activity.

Bacillus cereus SDK2 grown under the above mentioned conditions was also shown to have α-glucosidase activity (data not shown).

The optimum temperature and pH for pullulanase activity were 60°C and 7 ($p<0.05$), respectively (Figures 2 and 3). The study of the effects of different ions and reagents on the crude enzyme supernatant showed that only Ca^{+2} ions increased enzyme activity ($p<0.05$) (Tables 1 and 2).

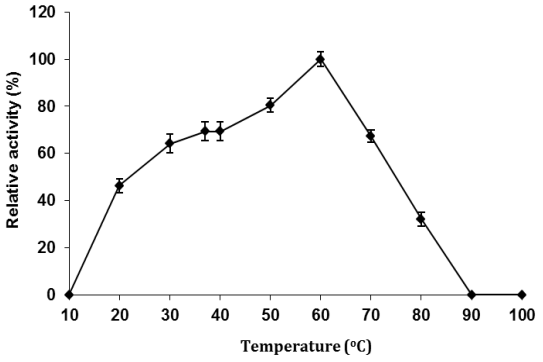

Figure 2. Effect of various temperatures on pullulanse activity of *B. cereus* SDK2. Optimal temperature was determined at selected temperatures ranging from 10 to 90°C. Data in this study represent the mean ± SD for three independent experiments, and error bars represent the SD. P-value of <0.05 was considered significant.

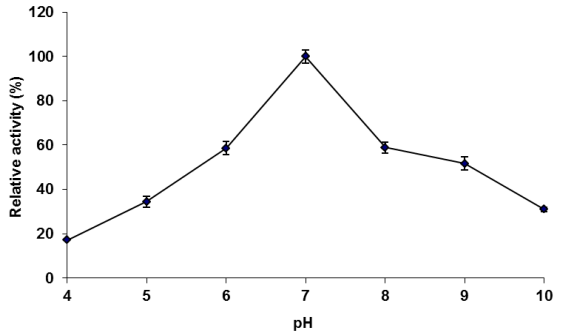

Figure 3. Effect of pH on pullulanse activity of *B. cereus* SDK2. Optimal pH was determined by measuring the enzyme activity in buffers of different pH ranging from 4–10; sodium citrate (pH 4), phosphate citrate (pH 5-6), sodium phosphate (7-8), glycine-NaOH (9-10). Data in this study represent the mean ± SD for three independent experiments, and error bars represent the SD. P-value of <0.05 was considered significant.

Table 1. Comparison of the effect of metal ions on pullulanase activity in *B. cereus* SDK2

	Relative activity (%) at two different concentrations	
Reagent	0.2mM	2mM
Mg^{+2}	42.5	31.8
Ca^{+2}	110	155.4
Zn^{+2}	45	34
Cu^{+2}	13.5	8.5
Fe^{+3}	78.8	42.5
Ni^{+2}	70.5	52.5
Control*	100.0	100.0

*The control had no metal ions and showed 100% activity. In all cases the results were statistically significant when p-values reported less than 0.05 ($P<0.05$).

Table 2. Comparison of the effect of reagents on pullulanase activity in *B. cereus* SDK2

	Concentrations	Relative activity (%)
EDTA	10mM	30.0
SDS	1mM	0
Urea	0.1M	90.0
	3M	40.0
	7M	25.0
Ammonium sulfate	0.5M	60.0
Control*	-	100.0

*The control had no reagents and showed 100% activity. In all cases the results were statistically significant when p-values reported less than 0.05 ($P<0.05$).

TLC analysis showed panose as the end-product of pullulan degradation by *B. cereus* SDK2, which confirmed that the extracellular enzyme produced by *B. cereus* SDK2 was a neopullulanase (Figure 4). *Bacillus cereus* has been identified as bacterium that is capable of producing pullulanases [7]. In fact, many pullulanase-producing bacteria have been isolated from soils containing starch and related polysaccharides, or other extreme environments such as hot springs [7, 18]. The neopullulanase from the *B. cereus* SDK2 strain of this study was found to have optimum activity at pH 7 and 60°C, a temperature important for the use of pullulanases in the saccharification process. In fact, Tang and colleagues have also reported a similar thermo-stable neopullulanase, isolated from soil sediments of a hot spring in Thailand [18]. The neopullulanase had maximum activity at a temperature of 75°C and a pH of 7. The enzyme was found to be stable over a wide pH range (3 to 9), maintaining more than 60% of the

enzyme activity following incubation at 60°C, for 1 h. However, their approach to isolation of this enzyme involved molecular methods involving the extraction of genomic DNA from soil samples, rather than cultivation and screening [18]. Their methods of choice were based on the rationale that many uncultivable microbes present in the environment are capable of producing unique products highly useful to academia, biotechnology and the food and pharmaceutical industries [18, 19].

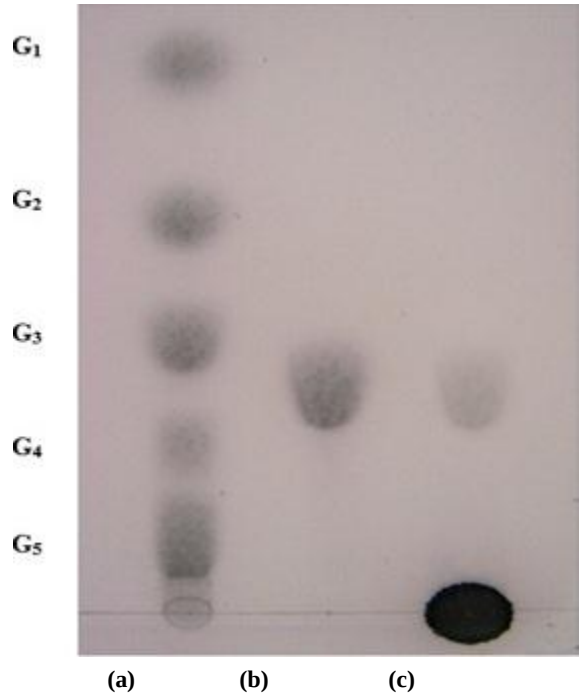

Figure 4. Product profile of the pullulan-degrading enzyme of *B. cereus* SDK2 using TLC: (a), G_1: Glucose, G_2: Maltose, G_3: Maltotriose, G_4: Maltotetraose, G_5: Maltopentaose. (b), Panose. (c) Product from *B. cereus* SDK2.

In this study, pullulanase-producing bacteria were isolated from samples (including soil) taken from industrial sites that used starch-related substrates. These included the flour, distilling and gluten industries. Following the isolation and screening procedures, a notable and repeatable observation was made, which showed that pullulanase-producing bacteria could only be isolated from the flour factory, and were not detected in samples taken from the other two industrial sites. This is in agreement with a previous finding reported by Moradi and colleagues who sampled the same three industrial sites on a separate occasion [20]. The main substrate and cereal grain used in the flour factory is wheat. These observations are yet another example of how environmental conditions can affect and influence the prevalence of certain microbial communities.

The next phase of the study demonstrated that the production of the neopullulanase enzyme was found to increase to a maximum of 13.43 U/ml near the end of logarithmic phase of growth, thereafter remaining constant. The curves obtained for bacterial growth and enzyme activity show that SDK2 growth and enzyme production are directly associated and occur in parallel, and almost simultaneously, reflecting the observations made by Saha and colleagues concerning the growth of *Thermoanaerobacter* strain B6A and its total pullulanase activity [4].

Results obtained also showed that Ca^{+2} ions increase pullulanase activity in the SDK2 strain, which is also in agreement with the results of Ling et al. [9], but is in contrast to those of Duffner et al. [10] who observed that although other metal ions and reagents inhibited pullulanase activity, Ca^{+2} and Mn^{+2} ions were found to have no effect on its activity. They had also suggested that the somewhat inhibitory effect of EDTA on pullulanase activity was likely due to the inability of the molecule to chelate divalent cations that are necessary for thermo-stable pullulanases to be functional [10].

However, research carried out by Ling and colleagues [9] on the pullulanase of *B. cereus* H1.5, demonstrated that with the exception of Ca^{+2} and Mn^{+2}, all other divalent metal ions inhibited enzyme activity significantly. In fact, as in our research, Ling and colleagues also observed that Ca^{+2} caused a noticeable increase in enzyme activity, by up to 170%. Furthermore, optimal activity was reported at 55°C and pH 6.0, and the enzyme's thermo-activity and thermo-stability was found to increase in the presence of Ca^{+2} ions [9], which is also similar to the observations made for the *B. cereus* SDK2 strain of this study, where in the presence of Ca^{+2} ions, its neopullulanase was found to have optimum activity at 60°C, a temperature important for the use of pullulanases in saccharification and other industrial processes (Table 1).

A great deal of research has been performed in recent years to isolate amylolytic microorganisms that are thermophilic or hyper-thermophilic [21, 22, 23]. However, there is a continuous demand for yet more efficient enzymes required for the starch processing, food, detergent, medical and pharmaceutical industries A diverse group of pullulan-degrading enzymes exist in nature that have numerous biochemical properties and possess many different mechanisms to degrade carbohydrates. Accordingly, such properties result in the generation of an extensive array of products that can be of significant use in various enterprises that include biotechnology and the medical, pharmaceutical, chemical and food industries [24, 25].

The presence of highly thermo-stable amylolytic enzymes is an absolute necessity in the two-step process of starch hydrolysis [22, 23]. In fact, the use of thermo-stable neopullulanase, together with other types of debranching enzymes and α -glucosidase are highly beneficial to the starch processing industries. In addition, the use of alkali-resistant forms of neopullulanase has also been reported in the detergent

industry. Moreover, the production of panose by neopullulanase can also be regarded as another major advantage of this enzyme. Panose has been identified as a new candidate for prebiotics [26]. In fact, it has been established that a diet supplemented with probiotic bacteria and prebiotic carbohydrates such as the α-glucoside, panose, can have a significant and positive impact on the human gastrointestinal tract [27, 28]. Additionally, panose is also applied to the prevention of dental caries. Because panose is slightly sweet and cannot be fermented by the bacteria residing in the oral cavity, it can be used as an anti-cariogenic sweetener in various foods. Panose inhibits the reaction that involves the formation of insoluble glucan from sucrose, thus preventing the adherence of bacteria to the teeth surface [29, 30].

4. Conclusion

As demonstrated in this study, the production of high levels of a new thermo-stable neopullulanase enzyme by *B. cereus* SDK2 that can also produce panose, together with its ability to grow at a wide range of temperatures and pH values, emphasizes the importance and significance of this bacterium in future applications to various enterprises that include biotechnology, the medical, food, detergent and pharmaceutical industries.

5. Acknowledgment

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6. Declaration of interest

All institutional or corporeal affixations of mine and all funding sources for the study are acknowledged. I certify that I have no commercial association that might represent a conflict of interest in connection with the submitted manuscript.

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