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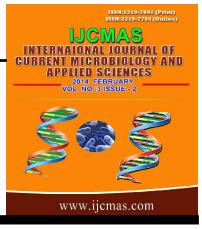

Original Research Article

Screening and isolation of powerful amylolytic bacterial strains

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A B S T R A C T

Several bacterial strains were isolated from three relevant industrial sites that were associated with the production of flour, gluten and non-alcoholic beer. Acquiring amylolytic strains with high activity among the isolated strains required a suitable systematic screening method. Our screening procedure consisted of three main steps that were based on the diameters of clear zones produced on different solid agar media containing specific substrates. In the first step, soluble starch was used as the primary substrate, and then the isolates that showed clear zones were selected as the amylolytic strains. Consequently, the next steps in the screening procedure led to the isolation of high amylolytic enzyme-producing strains, which were subsequently identified as, *Bacillus cereus*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis* and *Paenibacillus lautus* via morphological and biochemical tests and 16S rRNA sequencing. A phylogenetic tree was also constructed for alpha-amylase protein sequences associated with these four isolated strains. This procedure could be considered as a powerful systematic method for isolating of amylolytic microorganisms such as fungi. A significant observation made in this study was the distribution of the amylolytic bacterial strains in the three mentioned industrial sites. Accordingly, the probability of pullulan-degrading strains was found to be much less in the distillery, while there was a significant and common distribution for other sites regarding degradation of starch and related substrates. This study is a demonstration of how the environment determines the dominance of certain carbon-consuming communities.

Keywords

Screening;
Isolation;
Phylogenetic;
Bacteria;
Amylase;
Pullulan

Introduction

Amylolytic enzymes have been classified into the families of enzymes that break down starch and related polysaccharides by hydrolyzing their alpha-glycosidic bonds. The most important amylolytic enzymes include alpha-amylase, beta-amylase, glucoamylase, alpha-glucosidase and the pullulan-degrading enzymes (Vihinen and Mäntsälä, 1989). Due to the availability of substrates such as starch,

pullulan and glycogen as important sources of energy, many microorganisms, plants and animals, have evolved to produce a large variety of amylolytic enzymes that can hydrolyze such sources of carbon. Regarding the complex structure of starch and related oligo- and polysaccharides, starch-degrading organisms have to produce a relevant combination of starch-hydrolyzing- and related enzymes (Legin *et al.,* 1998; Bertoldo and Antranikian, 2002). These enzymes are generally known as amylases, which act on one type of substrate that consists of glucose residues linked through α-1-1, α-1-4 or α-1-6 glycosidic bonds, resulting in diverse products including dextrins, and progressively smaller polymers composed of glucose units (Windish and Mhatre, 1965).

Amylolytic enzymes are widely distributed among microbes. They have been extracted from several fungi, yeasts and bacteria, however, enzymes from fungal and bacterial sources have dominated applications in industrial sectors. *Bacillus* and *Aspergillus* spp. are known as the most active amylase producers. With the advent of new boundaries in biotechnology, the amylase family of enzymes and particularly those from microbial sources, have found potential application in a number of various fields, such as analysis (glycogen determination), industries (including brewing, baking, starch processing, detergent, textiles, paper, food) and clinical or pharmaceutical applications (diagnostic aids) (Pandey *et al.,* 2000; Gupta *et al.,* 2003).

The major advantages of using microorganisms for the production of amylases are their economical bulk production capacity and stability with respect to pH and temperature. Furthermore, microbes are easy to manipulate in regard to obtaining enzymes with the desired characteristics. Amylolytic enzymes play an important role in the degradation of starch, and are produced in bulk from microorganisms, representing about 25–33% of the world enzyme market (Ramesh and Lonsane, 1990; Konsula and Liakopoulou-Kyriakides, 2004; Sivaramakrishnan *et al.,* 2006). In the case of energy costs, worldwide interest has been growing in recent years regarding the acquisition of desirable amylases that are capable of digesting raw starches during the process of starch liquefaction, but without the need for heating, inevitably leading to the effective utilization of biomass and reduction in the total cost of starch processing (Wang *et al.,* 1999; Robertson *et al.,* 2006).

The microbial amylases that are used for industrial purposes are derived mainly from *Bacillus licheniformis*, *Bacillus amyloliquefaciens* and *Aspergillus oryzae*. Accordingly, native amylolytic microbial strains especially, have abundant applications to relevant industries in biotechnology (Nigam and Singh. 1995; Crabb and Mitchinson. 1997).

The aim of this study was to screen and isolate different kinds of strong amylolytic bacterial strains with the ability to produce a variety of desirable amylolytic enzymes, using a systematic, low cost and reliable screening procedure. By using this procedure, we were able to isolate the most important and strongest amylolytic bacterial strains, which can have significant roles in biotechnology as well as in other relevant researches. An investigation was also carried out in connection with the distribution of the

isolated amylolytic bacterial strains from the three industrial sites (flour, gluten and distillery factories).

Materials and Methods

Sampling and isolation of bacterial strains

Samples were collected aseptically from various parts of three relevant industrial sites in Tehran that were associated with the production of flour, gluten and non-alcoholic beer. Serial dilutions of the samples were spread onto Luria Bertani (LB) agar plates for separation of bacterial isolates. After incubation of plates at 37^{o}C for 48 h, the resulting colonies were transferred onto separate LB agar plates.

Screening procedure

Acquiring amylolytic strains with high activity among the obtained bacterial isolates required a suitable systematic screening method. Our screening procedure had three main steps that were based on the diameters of clear zones produced on solid agar media containing specific substrates. In the first step**,** approximately similar amounts of cells were transferred with a sterile tooth pick to specific media, which comprised soluble starch as the primary substrate. The isolates were then incubated at 37 C^{o} for 48 h and plates were overwhelmed with Lugol's reagent (Iodine-KI reagent) to test for enzyme hydrolysis as indicated by formation of clear zones around the colonies. Every colony that showed a clear zone, was selected as an amylolytic isolate. In the second step**,** soluble starch, potato starch and amylopectin were used as sources of carbon. In fact, the majority of the amylolytic microbial strains usually have good activity on these substrates. So, the plates were flooded with lugol's reagent (Iodine-KI reagent) and the diameters of clear zones were accounted for every one of the isolated strains. One third of the isolates with larger diameters were then selected for the third step that involved incubation on amylose agar and pullulan agar media (5% (w/v)), in addition to media from the first step. Utilization of these new media helped to screen for the presence of debranching enzymes in these isolates. Also the repetition and use of the second step media helped to reduce experimental error arising during the transfer of cells to the selective media. The pullulan agar plates were flooded with ethanol and incubated at room temperature for 2-4 h in order to detect pullulanase activity. The diameters of clear zones were accounted for every one of the isolated strains, and via their comparison, the best strains were isolated. Consequently, the screening procedure led to the isolation of the best and strongest amylolytic bacterial strains.

Substrate solubility

For solubilizing amylose (MERCK) (100 mg/ml from potato), 1 ml of ethanol was added to wet the amylose sample, followed by the addition of 10 ml of DW and 2 ml of 10% NAOH. The resulting mixture was warmed in boiling water until it was dissolved and then brought up to volume with DW. Its pH was then adjusted to 7.2 using a pH meter. The other substrates, soluble starch (MERCK), potato starch (MERCK), amylopectin (MERCK) and pullulan (MECRK) were soluble in water.

Identification of strains

Four amylolytic strains were finally selected and identified via morphological,

biochemical and molecular (16S rRNA) methods.

Biochemical tests

50 carbohydrate tests were carried out for every one of the 4 strains by means of the API kit (50 CH B, Biomeriux, France).

Molecular methods

Genomic DNAs of the selected strains were extracted using a DNA extraction kit (MERCK) and employed as templates in PCR reactions using the universal 16s rRNA gene primers, B1 (AGAGTTTGATCCTGGCTTAG) and B2 (TAAGAAGGTGATCCAGC) (26). The amplified 16S rRNA gene of each strain was sequenced on an automated DNA sequencer with the forward and reverse primers. For gaining the complete sequences of the 16S rRNA genes, Edit Seq (Hall, 1999) and Seq Man (Swindell and Plasterer, 1997) softwares were employed. Sequence similarities for the complete sequences of the 16S rRNA genes from different bacterial strains were determined via Blast analysis (Altschul *et al.,* 1997) available in the NCBI database. By means of these methods, the 4 strains were identified as *Bacillus cereus, Bacillus amyloliquefaciens, Bacillus licheniformis* and *Paenibacillus lautus*. These four new native bacterial strains were then registered in the EMBL databank under the accession numbers FN687449, FN678352, FN677986 and FN677987, respectively.

Phylogenetic tree

A phylogenetic tree was constructed to show similarity among the alpha-amylases of *B. cereus, B. amyloliquefaciens, B. licheniformis* and the most famous fungal alpha amylase producer, *Aspergillus oryzae*, according to databases NCBI, EMBL and UNI PROT by means of the MEGA4 software (Tamura *et al.,* 2007).

Determination of total reducing sugars or dinitrosalicyclic acid (DNS) method

This procedure determines the production of reducing sugars as a result of amylases action on starch based on the reduction of 3,5-dinitrosalicylic acid (DNS), in alkaline medium and at 80 C°, forming gluconic acid and the 3,5-diaminosalicylic acid, which is then measured with a spectrophotometer at 480 nm (Miller, 1959). The main flaw in this assay is a slow loss in the color produced and the destruction of glucose by constituents of the DNS reagent. To solve this limitation, a modified method for the estimation of reducing sugars was developed. Rochelle salts (sodium potassium tartrate) was excluded and 0.05% sodium sulphate was added to prevent the oxidation of the reagent (Michael, 1988).

Alpha-amylase activity

A reaction mixture containing 1ml of 0.1M sodium acetate buffer (pH 5.0) and 1ml of 0.5% (w/v) soluble starch solution was mixed and pre-incubated at 50 ° C for 10 min before adding an appropriate amount of the supernatant as an enzyme source. The buffer contained 7.5 mM Ca^{2+} ions. After 5 min, the reaction was terminated by adding 1 ml of 0.5 M HCl. The unhydrolyzed starch in this aliquot was estimated by the iodine method as described below. One unit of alpha-amylase activity was defined as the amount of enzyme that hydrolyzes 1mg of soluble starch in 1 min under relevant conditions (Ceska *et al.,* 1969).

Iodine method for starch estimation

An aliquot of the sample (starch hydrolysate) was mixed with 1ml of iodine reagent in a total volume of 3 ml DW. This reagent contained 0.02% (w/v) iodine and 0.2% (w/v) KI in 0.5 N HCl. To this mixture, 5ml of DW was added and the color that developed was read at 590 nm against an appropriate blank. The amount of starch was estimated using a standard soluble starch (MERCK) curve prepared under the same conditions (Ceska *et al.*, 1969).

Results and Discussion

Samplings were carried out from several sections of three industrial sites (flour, gluten and distillery factories). Ultimately, using the isolation procedure, about 488 bacterial isolates were secluded, the frequencies of which in the three sites included approximately 244 isolates from the flour factory, 118 isolates from the distillery and 126 isolates from the gluten factory.

Protein sequences associated with the alpha amylase enzymes concerning the above mentioned microbial strains were gathered from databases, and by similarity a phylogenetic tree was constructed (Fig.1). Results showed that, though *A. oryzae* is a eukaryotic organism, its alpha-amylase protein sequences are highly similar to bacterial ones, especially to *B. cereus*, with the main similarity residing in the 200-400 nucleotide region that in fact holds the active site.

The results associated with the formation of clear zones by the four final bacterial strains on specific substrates are shown in Table 1. It should be noted that, it is not necessary to choose strains with the exact diameter of clear zones that have been mentioned in the screening method (Fig. 3), and in reality it depends on your goal. For example thermophilic or alkalophilic strains may not be able to show such clear zones on these solid media.

Consequently, following the comparison of substrates and the resulting formation of clear zones, amylopectin was considered as the most suitable substrate for all amylolytic isolates which showed the highest enzyme activity and productivity in the presence of this substrate. Isolates produced the largest clear zones on agar containing amylopectin, which were surprisingly larger than on those containing the substrate amylose that has a simpler structure than amylopectin.

Hence, it can be suggested that to consider the total capability in amylolytic enzyme productivity of the isolates, among the carbon sources available, amylopectin can be recommended as the substrate of choice. However, it should be noted that starch was used in the first steps of the screening procedure because it was more economical.

The screening procedure that has been designed in this study is a systematic, low cost and convenient method for isolating powerful amylolytic bacterial strains with ability to produce a variety of desirable amylolytic enzymes, among primary bulky isolates (Fig.2). Although, this is a qualitative procedure based on the diameters of clear zones produced on different solid agar media containing specific substrates, but according to the complementary quantitative experiments, such as the DNS test (Miller, 1959) for the total amylase assay or alpha amylase assay (Ceska *et al.,* 1969) that, were carried out randomly for some of the isolates and for the final isolated amylolytic strains, this screening procedure is a reliable and precise method.

In fact, all assays verified the results of the qualitative procedure (data not shown).For this screening procedure, a significant community of primary isolates was required. In fact, the primary community consists of approximately 500 different bacterial isolates from various locations of three different industrial sites.

To date, no systematic procedure for isolating specific amylolytic bacterial strains has yet been reported, but certain attempts regarding total isolation have been carried out (Dhawale *et al.,* 1982; Castro *et al.,* 1993; Kristensen *et al.,* 1999; Sinead *et al.,* 2006), however, they are not sufficient for finding the desirable bacterial strains.

As mentioned before, the first step is crucial in recognizing the bacterial isolates as amylolytic microorganisms, with the ensuing steps involving further investigations of the amylolytic isolates (Fig.2). It has been shown that, there is a significant relationship between clear zone formation by amylolytic isolates on media containing specific solid substrates and the ability to produce the amylolytic enzymes. In fact, larger clear zones indicate higher amylolytic enzyme productivity (Dhawale *et al.,* 1982**;** Castro *et al.,* 1993).

In the second step, soluble starch-, potato starch- and amylopectin agar were employed, because the majority of the amylolytic microbial strains usually have good activity on these substrates. Actually, in such media, microbial strains (bacteria and fungi) would show their maximal potential in secretion of a variety of amylolytic enzymes, because they are specific sources of carbon and energy. One third of the strains, with clear zones of larger diameter, were then selected for the third step that involved incubation on amylose agar and pullulan agar media, in addition to media from the former step. Utilization of these new media helped to screen for the presence of debranching enzymes in these strains. Also, the repetition of the first step media helped to reduce experimental error arising during the transfer of cells to the selective media. The second and third steps were, in effect, compared with each other. In other words, the second step helps to find bacterial isolates with higher amylolytic activity and the third step helps to choose isolates with desirable debranching enzymes. From observation of the final steps, it can be suggested that clear zone formation on pullulanase agar could indicate the presence of isolates possessing enzymes with pullulan debranching activities, such as pullulanase types I, II and III. Clear zone formation on amylose agar medium has to be considered in light of the data obtained from incubations on starch agar and amylopectin agar and the difference may infer that there are highly likely to be debranching enzymes.

Interestingly, red or red-white clear zones on starch agar and amylopectin agar definitely indicate the sole production of the alpha-amylase enzyme by the isolates because dextrin, responsible for the red color, is one of the products of alpha amylase activity on starch. But the transparent clear zones on such media can infer the existence of debranching enzymes along with alpha-amylase, or the production of other major amylolytic enzymes with debranching activity, such as glucoamylase.

All the results in this study are tentative, which can be obtained by repetition of experiments, and further confirmed by additional quantitative procedures.

Fig.1 Phylogenetic tree of the alpha amylase protein sequences from *B. cereus, B. amyloliquefaciens, B. licheniformis* and *A. oryza*

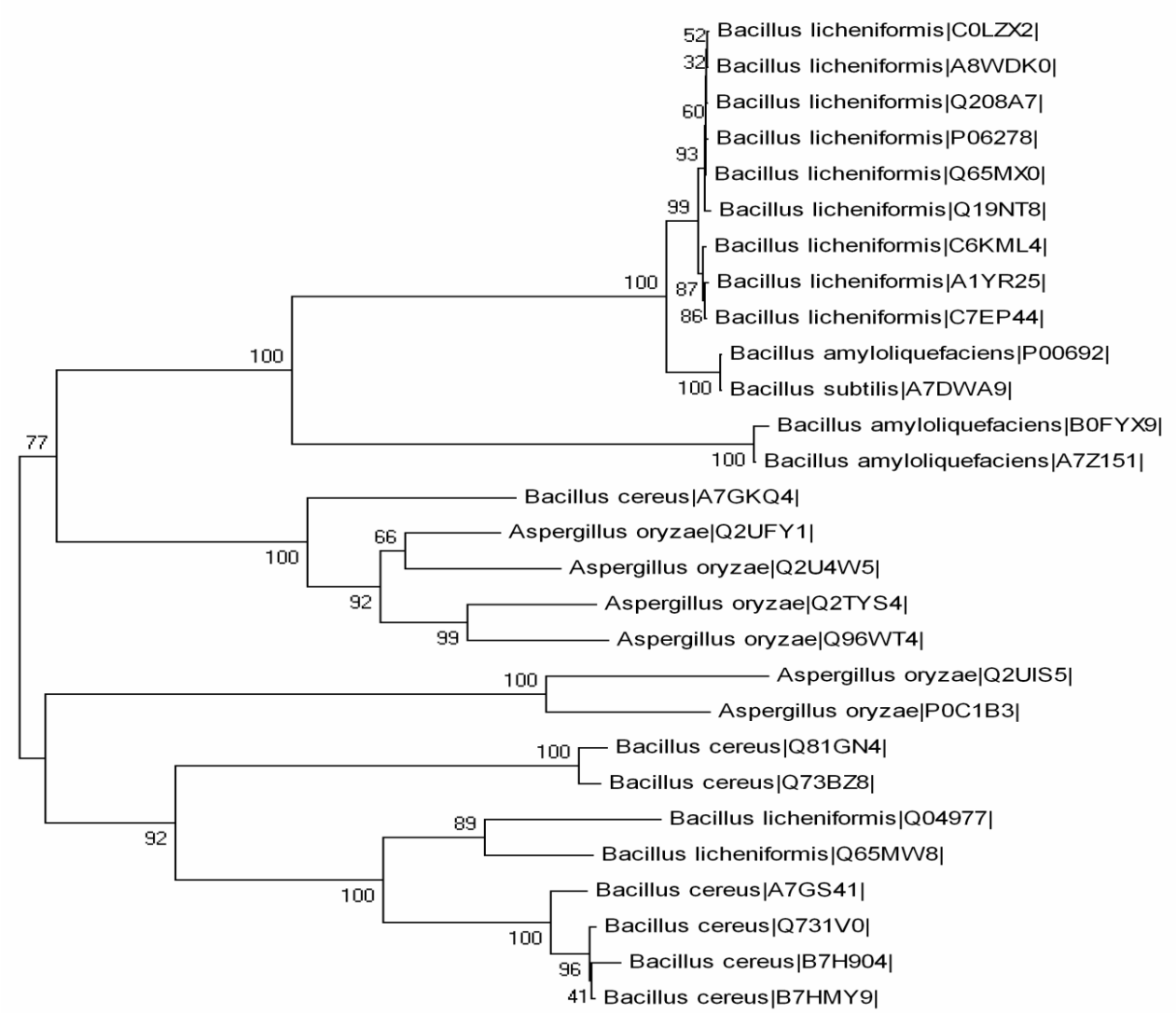


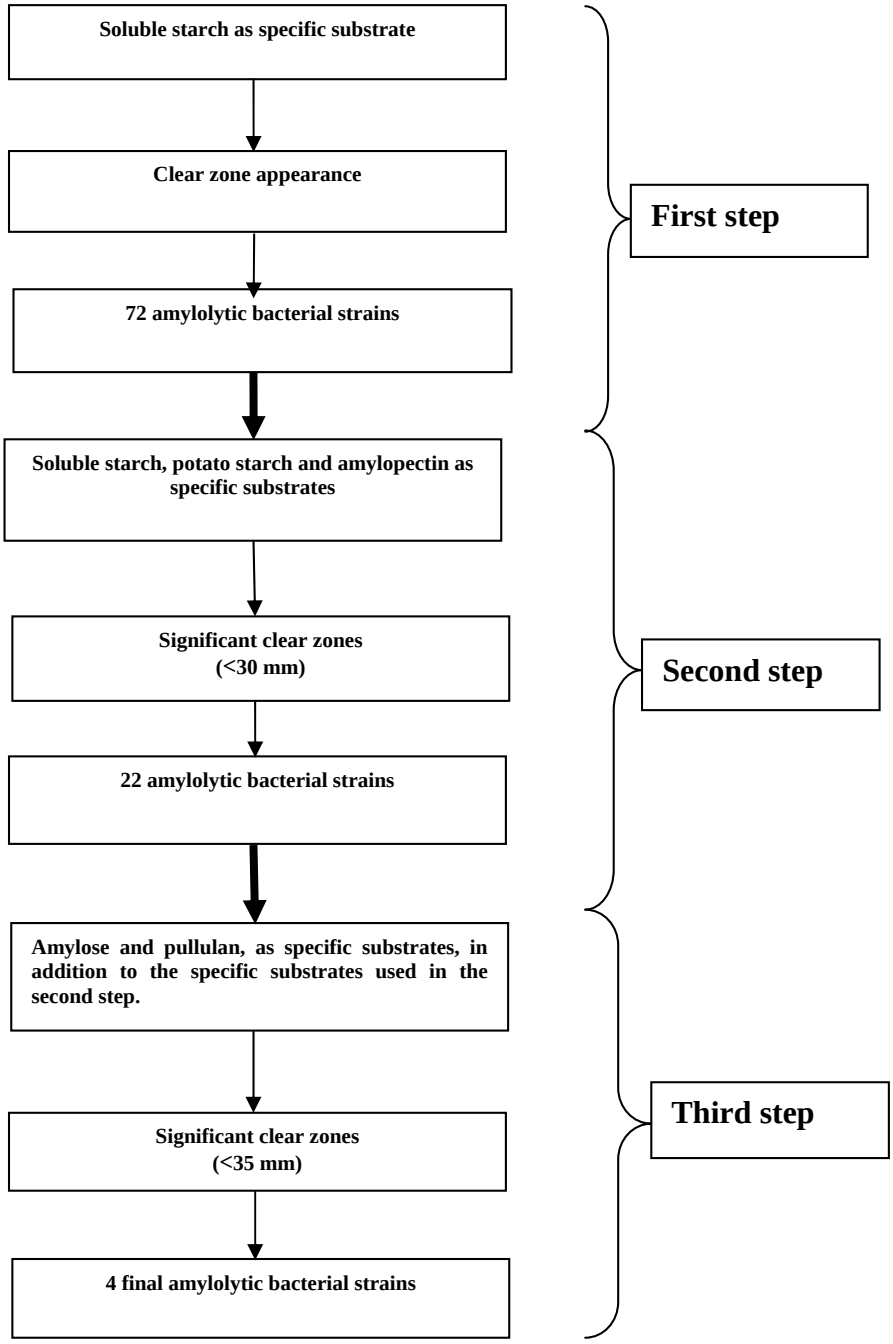
Table.1 Clear zone formation by the four final amylolytic bacterial strains on different substrate-agar media

Specific substrate	*Bacillus cereus*	*B. amyloliquefaciens*	*B. licheniformis*	*P. lautus*
Soluble Starch Agar	++++	++++	++++	++++
Potato Starch Agar	+++	++	+++	++
Amylopectin Agar	+++++	+++++	++++	++++
Amylose Agar	+[a]	++++	++++	+++
Pullulan Agar	+++	-[b]	-	++++

[a] Each (+) indicates approximately one centimeter diameter clear zone

[b] (-) indicate no clear zone production on substrate-agar plates

Fig.2 Three steps of the entire screening procedure



One of the advantages of this systematic procedure is its flexibility with regard to isolation of amylolytic strains based on their thermal and pH characteristics. Dependent on the aims and type of strains, different incubation temperatures can be used in all the three screening steps. For example in order to isolate mesophilic and thermophilic strains, the incubation temperatures can be set at between 50-60 ^{o}C and 90-100 ^{o}C, respectively. Also, for isolation of alkalophilic and acidophilic bacterial strains, pH of the media containing specific substrates, can be adjusted to 9-11 and 4-6, respectively.

The other remarkable observation made in this study was the distribution of the amylolytic bacterial strains in the three mentioned industrial sites. Accordingly, the probability of pullulan-degrading strains was found to be much less in the distillery, while there was a significant and common distribution for other sites regarding degradation of all the above mentioned substrates.

For all cereals, limit dextrinase (EC 3.2.1.142) also known as Re-enzyme or plant pullulanase, is one of the vital enzymes, which has a complementary role in the germination process and debranching activity during degradation of starch to glucose. The degradation of starch by α- and β- amylases present in germinated cereal grain results in the accumulation of branched dextrins, since neither amylase has any effect on α-1, 6-D- glucosidic linkages. The role of the limit dextrinase is hydrolysis of these linear dextrins that can be subjected to further amylolytic attack.

Some evidences have predicted that the main cereals, consisting of wheat, barley, maize, sorghum and rice have in effect been derived from a joint ancestral grass some 60 million years ago (Moore *et al.*, 1995).

However, the pullulanase (limit dextranase) enzyme in barely (the basis of the distillery industry), by comparison with other cereals, has been found to have a much lower activity (McCleary, 1992). Some possible causes for lower pullulanase activity in barley as compared to other cereals have been discussed in several articles. It has been confirmed that there is a protein in barley that inhibits limit dextranase. It has also been shown that over-expression of thioredoxin in barley greatly enhances the level of pullulanase enzyme expressed in the barley (Cho *et al.*, 1999; Jones and Budde, 2003). In fact, researchers have concluded that stimulation of this enzyme could not be traced to the reduction and inactivation of the inhibitor, and it has been suggested that the mechanism is either an increase in the *de novo* synthesis of the enzyme or by a promotion of the release of the enzyme from binding (Craig and Bamforth, 2008).

Pullulanase enzymes, as the debranching enzymes found in certain bacterial genera, are crucial to the hydrolysis of α-1-6 bonds in complicated components such as starch or amylopectin. Interestingly, it has been shown that, there is approximately 40-50% identity among the pullulanase protein sequences of barley and some bacterial genera, such as *Klebsiella* (Kristensen *et al.*, 1998; Kristensen *et al.*, 1999).

In consideration of the fact that, there is 50% sequence similarity in the pullulanase gene of certain bacteria and barley, it can be deduced that the abovementioned inhibiting factors may also affect the activity of pullulanase in bacteria that reside at sites where barley is present.

In order to achieve a better consideration and confirmation of this unique observation, future work is required to take samples from similar sites where the substrate barley is present.

Can such a factor (as mentioned above) or factors affect the activity of the bacterial pullulanase? It is hoped that such future work will help to understand the reasons behind this unique observation.

In conclusion, the screening procedure developed in this research is a powerful systematic approach towards isolation of highly active amylolytic bacterial strains. The procedure is also flexible and cost effective, which can be used for the effective screening and isolation of highly active microbial strains, including fungi. This study is a demonstration of how the environment determines the dominance of certain carbon-consuming communities. Accordingly, these results show that the presence or absence of activity towards specific substrates by the isolated amylolytic bacterial strains may have a significant relationship with the origin of the strains.

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