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Screening of Xylanase and Glucose Isomerase Producing Bacteria Isolated from Hot Springs in Turkey

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ABSTRACT

The aim of this study was screening of xylanase and glucose isomerase producing thermophilic bacteria isolated from some hot springs located in Aegean Region of Turkey. Total sixty eight thermophilic isolates (*Anoxybacillus, Brevibacillus, Geobacillus, Aneurinibacillus, Thermus, Paenibacillus* and *Proteobacter*) were collected previously from these fields and identified based on 16S rDNA gene sequences. Isolates were screened by plate assay for determining the xylanase and glucose isomerase production abilities seperately in order to find new strains for industrial processes. After an incubation period of two days for xylanase and 5-6 days for glucose isomerase at 50-60 °C, positive isolates were determined. Enzyme producing isolates were confirmed by spectrophotometric measurements with crude enzyme extracts, birchwood xylan and glucose were used as substrates. Most of the isolates (fifty nine) were positive for xylan degradation while only sixteen of them were positive for glucose isomerase activity. Fourteen of the isolates showed both xylanase and glucose isomerase activity. None of the isolates belong to the genera *Paenibacillus, Aneurinibacillus,* and *Proteobacter* were glucose isomerase positive, although the glucose isomerase activity of *Geobacillus* isolates were notably high. Both xylanase and glucose isomerase activities were observed at 50-60 °C which is suitable for biotechnological applications.

Keywords: Birchwood xylan, Glucose isomerase, Thermophilic bacteria, Xylanase

1. INTRODUCTION

Microbial enzymes have widespread uses in industries because of their biocatalytic potential for a large number of reactions. Microbial enzymes show higher activity and stability than plant and animal enzymes at high temperatures. Microbial enzymes can also be cultured in large quantities in a short time by fermentation [1]. With the recent developments in biotechnology, important tools for the efficient development of new enzymes have provided. Thermophilic enzymes have especially been topics for much research because of their stability at high temperatures which allowing high substrate solubility, better mixing, more mass transfer rate, and less risk of contamination [2, 3]. These enzymes are not only more thermostable, but also more resistant to extreme pH values, detergents, the other organic solvents and denaturing agents than their mesophilic counterparts what makes them extremely interesting for industrial processes [4, 5, 6]. The interest in microbial enzymes is increasing rapidly in recent years, the principle industrial sectors being in the food industries, starch processing, detergent, paper industry etc. We have focused on two of the industrial enzymes in this work that have great importance in food and paper industry; xylanases and glucose isomerases.

Xylan is considered as the second widely available polysaccharide in nature after cellulose [7]. It is located mainly in the secondary cell wall of annual plants and hardwoods and is thought to be form a linkage between lignin and other polysaccharides. The heterogeneous polysaccharide structure of xylan composed mostly of linear chains of D-xylose linked through β-1,4-glycosyl bonds [8, 9]. Because of its complexity and heterogeneity,

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several enzymes involved the complete degradation of xylan. Xylanases play a key role in this degradaditon [10]. Xylanase (E.C 3.2.1.8) degrades β-1,4- glycosidic linkages randomly, and produce xylose and xylo-oligosaccharides [11, 12, 13]. Xylanases have a great industrial importance, pulping and bleaching processes, food and feed industry, textile processes and waste treatment are the main applications of xylanase [14]. It is also used in preparation of xylooligosaccharides which are used as prebiotics and in combination with cellulase and pectinase for clarification of fruit juices [15]. Utilization of xylanase reduces the environmental pollution caused by chemicals. Microorganisms such as actinomycetes, fungi and bacteria are the main sources of xylanases [9]. Xylan producing microorganisms are widespread in the world and also reported in various extreme environments, such as thermal springs [16], marines [17], and Antarctic environments [18]. High temperatures are needed for most of the industrial applications that use xylanases, for this reason thermostable xylanases would be advantegous [19].

D-Glucose/xylose isomerase (D-xylose ketol isomerase; EC 5.3.1.5), commonly referred as glucose isomerase (GI), is one of the three highest tonnage value enzymes in industry, amylase and protease being the other two [20]. Glucose isomerases are used in industry to catalyze the reversible conversion of D-glucose to D-fructose on the first step of xylose metabolism in many microorganisms [21]. Interconversion of xylose to xylulose serves a nutritional requirement in saprophytic bacteria that living on decaying plant matter and also involves in the hemicellulose bioconversion for producing ethanol [20]. This isomerization reaction is important for the high fructose corn syrup (HFCS) production process from corn starch. HFCS is typically used as a sweetener in food industry. Until 1970s the main sweeteners of food industry was sucrose derived from sugar beet (40%) and sugarcane (60%). Glucose isomerase was firstly used in Japan for the production of HFCS and later in the United States and nowadays it becomes one of the most important industrial enzymes. High temperature and alkaline pH are needed in the enzyme process used in sweetener industry for conserving a higher concentration of fructose [22, 23]. The main sources of commercially available GI are mesophilic microorganisms that exhibit optimum activity at a pH range from 7.5 to 9.0 [24]. Isolating new thermophilic microorganisms that are capable of producing GI with high level of activity and stability at elevated temperatures will be of great benefit in the industrial production of HFCS.

The aim of this study was to detect new thermotolerant/thermophilic bacterial strains with the capability of the production of thermostable xylanase and/or glucose isomerases for industrial applications. This paper would be helpful for paper and food industries since it will provide new xylanase and GI producing thermophilic microorganisms.

2. MATERIALS AND METHODS

2.1. Bacterial isolates and culture conditions

Water samples were collected from Karakoc, Kaynarca and Nebiler Hot Springs (Izmir), Alangullu and Camkoy Hot Springs (Aydin), and Geotermal field of Omerbeyli (Germencik/ Aydin). All of 68 isolates derived from these samples were identified based on 16S rDNA sequences by Inan [25]. According to 16S rDNA sequence anaylses, 26 of the isolates belong to the genus *Anoxybacillus*. 15 *Brevibacillus*, 13 *Geobacillus*, 7 *Thermus*, 4 *Aneurinibacillus*, 2 *Proteobacter* and 1 *Paenibacillus* isolates were also identified. All of the isolates were cultured in Luria Bertani (LB) medium with an optimum growing temperature of 50-60 °C.

2.2. Xylanase activity assay

2.2.1. Plate assay

Xylanase activity was determined by the method of Gessesse and Gashe [26]. Single colonies from fresh LB agar plates were patched onto xylan included plates (1% xylan, 0.2% yeast extract, 2% agar, 0.5% peptone, 0.05% NaCl, 0.05% $MgSO_4$, 0.015% $CaCl_2$ and pH 7.0) and incubated for 48 hours at 50-60 °C. After incubation period plates were flooded with 0.1% Congo red for 15 min and by washing two to three times with 1 M NaCl the dye was removed. Transparent zones around colonies represents the degradation of xylan.

2.2.2. Spectrophotometric measurements

Fresh cultures of each isolate (2 ml) were inoculated into xylanase specific medium (g/L: 10.0 g xylan, 5 g pepton, 2 g yeast extract, 0.5 g MgSO4, 0.15 g $CaCI_2$ and 0.5 g NaCI, pH 7.5) for xylanase production. After centrifugation of 4500

x g for 5 min, supernatants were used as enzyme sources.

Dinitrosalicylic acid method was used for determining the xylanase activity by measuring the release of reducing sugars from Birchwood xylan [27]. 0.25 mL of enzyme sample was mixed with 0.75 ml of substrate (%1 solubilised birchwood xylan) in phosphate buffer, pH 6.5 and incubated at optimum bacterial growing temperature for 20 min. 1.0 mL of dinitrosalicylic acid (DNS) solution was added to the samples and boiled for 5 min. The absorbance was measured at 540 nm. The reaction was terminated at zero time in the control tubes. Solubilised xylan was prepared by stirring birchwood xylan with 0.5 M Tris-HCl (pH 8.0) buffer for overnight at room temperature. After centrifugation supernatant was used as substrate.

2.3. Glucose isomerase activity assay

2.3.1. Plate assay

The method of Lee et al. [28] was used for determination of glucose isomerase activity. Bacterial isolates were incubated in agar plates supplemented with 1% xylose for overnight. Fructose (2%), $MgSO_4$ (5 mM), $CoCl_2$ (0.5 mM), glucose oxidase (20 U/ml), peroxidase (4 U/ml), and benzidine (0.4 mg/ml) in 100 mM MOPS (morpholinepropane sulfonic acid) buffer (pH 7.0) were mixed with top agar (0.7%) at 50°C and poured on the colonies. The plates were incubated for 5-6 days at 50-60°C. Positive samples showed a dark brown color around the colonies.

2.3.2. Spectrophotometric measurements

Bacterial cells were incubated in LB medium supplemented with 0.5% xylose and harvested at the late-logarithmic phase by centrifugation at 13,000 rpm for 5 min, resusupended with 25 mM phosphate buffer (0.2 mg lysozyme, 5 µg DNase and 0.1% Triton X-100, pH 7.0) and lysed by incubating in a shaker for 3-4 hours. After a centrifugation period of 15,000 rpm. for 30 min supernatants were collected for enzyme activity assays [29].

Cysteine-carbazole-sulfuric acid method was used for estimating the amount of fructose or xylulose formed after the enzyme reaction. Based on this method, glucose isomerase activity was measured by incubating a reaction mixture that contained 10 mM MgSO4, 1 mM CoCl2, 0.5 M glucose, and the enzyme in 100 mM MOPS buffer (pH 7.0) at 55°C for 30 min. İncubation period followed by addition of 0.5 M perchloric acid for terminating the reaction. 1.5% cysteine hydrochloride and 0.12% carbazole added to the reaction mixture and mixed thoroughly, right after 70% sulfiric acid added, mixed and incubated at 60°C for 10 min. The absorbance was measured at 560 nm.

3. RESULTS AND DISCUSSION

In the present study, thermophilic isolates derived from different hot springs (formerly identified) were screened for xylanase and glucose isomerase activity.

3.1. Xylanase activity

Sixty eight isolates of bacteria were tested for xylanase production on xylan (1%) included agar plates for 2 days at 50-60 °C. Zone formation around the bacterial growth after addition of Congo red solution (0.1%) was identified for the xylanase positive isolates. Only nine of them were negative for xylanase activity based on plate assay. Five of the xylanase negative isolates belongs to the genus *Anoxybacillus*, three of them *Brevibacillus* and one of them belong to the genus *Geobacillus* (Table 1). Zone forming isolates were inoculated into xylan specific medium (10.0 g/L xylan) for xylanase production for further analyses. Dinitrosalicylic acid method [27] was used for spectrophotometric determination of xylanase activity. Spectrophotometric measurements at 540 nm with Birchwood xylan (%1) as substrate revealed that all 59 of the isolates were hydrolyse the xylan to xylo-oligosaccharide molecules. Especially *Anoxybacillus* TF15 and *Geobacillus* DF20, TF11, PDC9, PDC11, and TH2 exhibited a two fold activity than the other isolates with Birchwood xylan.

Xylanase producing bacterial strains were isolated from different kind of areas, such as Antarctic environments [18], marines [17], soda lakes [30] and thermal springs [16]. Microbial production of xylanases from bacteria, such as *Bacillus*, *Cellulomonas*, *Micrococcus*, *Staphylococcus*, *Paenibacillus*, have been reported [9, 31, 32, 33]. Because thermostability is a desired characteristic, thermostable xylanases have a great importance among industrial enzymes. An extreme thermophile *Rhodothermus marinus* producing xylanases active at 80 °C have been identified [34]. Some other thermostable xylanases for example *Bacillus spp., Thermotoga sp., Thermus*

sp., and *Streptomyces sp.* were also reported that active at 60-70 °C [35]. Xylanase producing thermophilic *Geobacillus* and *Anoxybacillus* strains were also reported [36, 37, 38, 39]. In this work, all of the *Thermus*, *Aneurinibacillus*, and *Proteobacter* isolates exhibited xylanase positive features. The only one *Paenibacillus* isolate included in this work was also degraded the xylan. Only a few of the *Anoxybacillus*, *Geobacillus* and *Brevibacillus* samples were negative for xylanase production.

3.2. Glucose Isomerase activity

Glucose isomerase activity was determined based on the method of Lee et al. [28]. All of the 68 thermophilic isolates were inoculated onto glucose isomerase specific plates. After an incubation period of 5-6 days, only 16 of the isolates have showed a dark brown color around colonies, which nine of them belongs to the genus *Geobacillus*, and the others include five *Anoxybacillus*, a *Brevibacillus* and a *Thermus* isolates (Table 1). Spectrophotometric measurements were carried out with cysteine-carbazole-sulfuric acid method for verifying the results. Among the sixteen isolates screened, five *Geobacillus* and an *Anoxybacillus* isolates showed maximum glucose isomerase activity. *Brevibacillus* and *Thermus* samples exhibited relatively poor activity.

Pseudomonas hydrophila was the first discovered microorganism that catalyse the isomerization of D-glucose to D-fructose [40], since then a large number of microbial species has been detected for glucose isomerase activity [41, 42]. *Lactobacillus* [43], *Streptomyces* [44], *Bacillus* [45, 46], *Arthrobacter* [47] are some of the glucose isomerase producing bacteria reported.
Paenibacillus, Aneurinibacillus and *Proteobacter* isolates used in this work did not exhibit glucose isomerase activity. There is also no evidence for *Aneurinibacillus* and *Proteobacter* genera about GI activity in literature, whereas D-xylose isomerase activity have been reported for *Paenibacillus* sp. strain by Moneke et al. [48]. Some of the isolates belong to the genera *Geobacillus*, *Anoxybacillus*, *Brevibacillus* and *Thermus* were positive for GI activity in our screening results. There is already some reports in literature about glucose isomerase activity of *Anoxybacillus* [49], *Geobacillus* [50], *Thermus* [51], and *Brevibacillus* [52] strains. Because of the industrial importance of Glucose isomerases, especially thermostable and acid-stable ones, further investigations are already under way.

Table 1. Production of xylanase and glucose isomerase by various bacterial isolates

Genus	Strain	Place (Hot Spring)	Glucose Isomerase	Xylanase
Anoxybacillus	PDF 1	Germencik/ Aydin	-	+
Anoxybacillus	PDF 2	Germencik/ Aydin	-	-
Anoxybacillus	PDF 3	Karakoç/Izmir	-	+
Anoxybacillus	PDF 15	Camkoy/ Aydin	-	+
Anoxybacillus	PDF 16	Camkoy/ Aydin	-	+
Anoxybacillus	PDF 18	Karakoc/Izmir	-	+
Anoxybacillus	PDF 21	Karakoc/Izmir	+	+
Anoxybacillus	PTF 26	Karakoc/Izmir	-	+
Anoxybacillus	PTF 37	Karakoc/Izmir	-	+
Anoxybacillus	PTF 38	Kaynarca/Izmir	-	+
Anoxybacillus	DF 1	Germencik/ Aydin	+	+
Anoxybacillus	DF 2	Germencik/ Aydin	-	+
Anoxybacillus	DF 3	Karakoc/Izmir	-	+
Anoxybacillus	DF 5	Kaynarca/Izmir	-	+
Anoxybacillus	DF 8	Kaynarca/Izmir	-	+
Anoxybacillus	DF 10	Kaynarca/Izmir	-	+
Anoxybacillus	DF 11	Kaynarca/Izmir	-	+
Anoxybacillus	DF 14	Alangullu/Aydin	-	-
Anoxybacillus	DF 15	Alangullu/Aydin	+	-
Anoxybacillus	DF 16	Alangullu/Aydin	-	+
Anoxybacillus	DF 17	Camkoy/ Aydin	-	+
Anoxybacillus	DF 18	Camkoy/ Aydin	-	+
Anoxybacillus	DF 19	Camkoy/ Aydin	-	-
Anoxybacillus	TF 15	Karakoc/Izmir	++	+
Anoxybacillus	TH 4	Nebiler/Izmir	-	-
Anoxybacillus	TH5	Camkoy/ Aydin	+	+
Brevibacillus	PDF 4	Camkoy/ Aydin	-	+
Brevibacillus	PDF 10	Camkoy/ Aydin	-	+

Brevibacillus	PDC 1	Germencik/ Aydin	-	+
Brevibacillus	PDC 2	Karakoc/Izmir	-	+
Brevibacillus	PDC 3	Alangullu/Aydin	-	+
Brevibacillus	PDC 4	Germencik/ Aydin	-	+
Brevibacillus	PDC 5	Nebiler/Izmir	+	+
Brevibacillus	PDC 6	Alangullu/Aydin	-	+
Brevibacillus	PDC 7	Kaynarca/Izmir	-	+
Brevibacillus	PDF 23	Germencik/ Aydin	-	-
Brevibacillus	PTF 25	Karakoc/Izmir	-	+
Brevibacillus	PTF 30	Karakoc/Izmir	-	-
Brevibacillus	PTF 33	Camkoy/ Aydin	-	+
Brevibacillus	PTF 40	Kaynarca/Izmir	-	+
Brevibacillus	PDF 41	Kaynarca/Izmir	-	-
Paenibacillus	PTF 34	Nebiler/Izmir	-	+
Aneurinibacillus	PDF 6	Alangullu/Aydin	-	+
Aneurinibacillus	PDF 13	Nebiler/Izmir	-	+
Aneurinibacillus	PDF 24	Nebiler/Izmir	-	+
Aneurinibacillus	PTF 32	Alangullu/Aydin	-	+
Proteobacter	PDF 20	Nebiler/Izmir	-	+
Proteobacter	PTF 31	Nebiler/Izmir	-	+
Geobacillus	DF 20	Germencik/ Aydin	++	+
Geobacillus	TF 11	Alangullu/Aydin	++	+
Geobacillus	TF 12	Karakoc/Izmir	+	+
Geobacillus	PDC 8	Kaynarca/Izmir	+	+
Geobacillus	PDC 9	Alangullu/Aydin	++	+
Geobacillus	PDC 10	Alangullu/Aydin	+	+
Geobacillus	PDC 11	Germencik/ Aydin	++	+
Geobacillus	PDC 12	Germencik/ Aydin	+	-
Geobacillus	PDC 13	Camkoy/ Aydin	-	+
Geobacillus	PDC 14	Karakoc/Izmir	-	+
Geobacillus	TH 2	Camkoy/ Aydin	++	+
Geobacillus	TH 3	Camkoy/ Aydin	-	+
Geobacillus	TH 6	Karakoc/Izmir	-	+
Thermus	TF 2	Germencik/ Aydin	-	+
Thermus	TF 3	Germencik/ Aydin	+	+
Thermus	TF 5	Nebiler/Izmir	-	+
Thermus	TF 6	Kaynarca/Izmir	-	+
Thermus	TF 7	Karakoc/Izmir	-	+
Thermus	TF 8	Kaynarca/Izmir	-	+
Thermus	TF 9	Germencik/ Aydin	-	+

4. CONCLUSIONS

In this study it is aimed to find out new thermophilic xylanase and/or glucose isomerase producing strains for industrial applications. For this purpose, formerly identified sixty eight thermophilic isolates collected from hot springs, located in the west side of Turkey, were screened for xylanase and glucose isomerase activity. Among sixty eight isolates, fifty nine of them exhibited xylanase activity which indicates that most of the bacteria derived from hot springs can degrade xylan. However only sixteen of the isolates showed glucose isomerase activity that is glucose degradation is not a common feature for these bacteria. Fourteen of the isolates were positive for both xylanase and glucose isomerase but further analyses should be done for determining that which substrate (xylan or glucose) is more preferable for these bacteria. The optimal enzyme activity of the isolates was between 50-60 °C, which make them potential tools for industrial applications.

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