

GROWTH TEMPERATURE OF DIFFERENT LOCAL ISOLATES OF *BACILLUS* SP. IN THE SOLID STATE AFFECTS PRODUCTION OF RAW STARCH DIGESTING AMYLASES

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Abstract - Natural amylase producers, wild type strains of *Bacillus* sp., were isolated from different regions of Serbia. Strains with the highest amylase activity based on the starch-agar plate test were grown on solid-state fermentation (SSF) on triticale. The influence of the substrate and different cultivation temperature (28 and 37°C) on the production of amylase was examined. The tested strains produced α-amylases when grown on triticale grains both at 28 and at 37°C, but the activity of amylases and the number and intensity of the produced isoforms were different. Significant hydrolysis of raw cornstarch was obtained with the *Bacillus* sp. strains 2B, 5B, 18 and 24B. The produced α-amylases hydrolyzed raw cornstarch at a temperature below the temperature of gelatinization, but the ability for hydrolysis was not directly related to the total enzyme activity, suggesting that only certain isoforms are involved in the hydrolysis.

Key words: *Bacillus* sp., α-amylase, isoform, raw starch hydrolysis

INTRODUCTION

Starch and its two main constituents, amylose and amylopectin, are degraded by α-amylases which are the major enzymes involved in the hydrolysis of (1→4)-α-D-glucosidic bonds. Alpha-amylases are very important in biological reactions such as fermentation, germination or digestion, but also widely used in industry e.g. in the production of glucose syrups, control of anti-staling in bread products or in detergents to remove starch-based stains (Lauro, 2001).

As native starch is water insoluble at room temperature, many applications of α-amylases are carried out at high temperature and pressure where the starch is gelatinized (Lauro, 2001). To reduce the cost of starch processing, the importance of enzymatic hydrolysis of raw starch without cooking has recent-

ly become well recognized (Wang et al., 2007). This has generated a worldwide interest in the discovery of several raw starch degrading enzymes (RSDE) that can directly degrade raw starch granules below the gelatinization temperature of starch (Sun et al., 2010).

RSDE are produced by plants (Georg-Kraemer et al., 2001; Ueda et al., 2008) and microorganisms (Demirkhan et al., 2005). In spite of the wide distribution of RSDE, microbial sources have many advantages for industrial production, such as cost effectiveness, consistency, less time and space required for production, and ease of process modification and optimization. Therefore, microbial sources are preferred for large-scale production. Screening for potent RSDE producers is a key step for RSDE production. These strains have been isolated mainly from rotting starchy material, soil and air. Among

them, *Aspergillus* sp., *Rhizopus* sp. and *Bacillus* sp. are apparently the most common choices (Sun et al., 2010).

Bacillus species continue to be dominant bacterial workhorses in microbial fermentations and some *Bacillus* sp. are on the Food and Drug Administration's GRAS (generally regarded as safe) list. The capacity of selected *Bacillus* strains to produce and secrete large quantities (20-25 g/L) of extracellular enzymes has placed them among the most important industrial enzyme producers (Schallmey et al., 2004).

Even though most enzymes are produced by means of submerged fermentation techniques, solid-state fermentation (SSF) has gained renewed interest from researchers for the production of enzymes due to its economic and engineering advantages. Solid-state fermentation is simpler, requires less capital, has superior productivity, lower energy requirement, requires simpler fermentation media, does not require rigorous control of fermentation parameters, uses less water, produces less wastewater, allows for the easy control of bacterial contamination, and has a lower downstream processing (Sivaramakrishnan et al., 2006). Earlier studies have reported alpha-amylase production by solid-state fermentation of *Bacillus licheniformis* M27 (Ramesh and Lonsane, 1990), *B. coagulans* (Babu and Satyanarayana, 1995), *B. amyloliquefaciens* (Gangadharan et al., 2006), *Bacillus* sp. PS-7 (Sodhi et al., 2005) and *B. subtilis* (Ozdemir et al., 2009) on different fermentation media. Because the bacterial production of amylase is affected by certain factors, including microbial strain, culture medium formula and physicochemical conditions, enhancement of target amylase productivity in any fermentation system could be achieved through the improvement of these factors (Park et al., 1997; Gangadharan et al., 2008). Currently, efforts are underway to explore approaches to enhance the efficiency of fermentation systems by using inexpensive agriculture residues as substrates (Rajagopalan and Krishnan, 2009; Cavalcante Barros et al., 2013), or by developing more efficient environmental conditions (Kiran and Chandra, 2008; Shivakumar, 2012). Tem-

perature is one approach that can be used to improve productivity in microbial fermentation (Park et al., 1997; Tanyildizi et al., 2007).

The aim of this study was to show that different growth temperature affects different raw starch digesting amylase isoform production when wild type *Bacillus* sp. was grown.

MATERIALS AND METHODS

Chemicals

All reagents and solvents used were of the highest available purity and at least of analytical grade. They were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Raw cornstarch was isolated in our laboratory according to the standard recommended procedure (Božić et al., 2013).

Selection, isolation and identification of microorganism

Different soil and milk samples were taken from different regions of Serbia. The soil used in all experiments was collected from a 10 cm-depth layer. Each gram of sample was suspended in 99 ml of sterile distilled water and shaken. The samples were heated at 80°C for 10 min in a water bath. Then the soil suspensions were plated on nutrient agar medium. The plates were incubated at 37°C for 24 h. The colonies that were found on the plates were transferred onto another nutrient agar consisting of 1% soluble starch. These plates were also incubated at 37°C for 24 h. Several amylase-producing bacterial colonies were selected after flooding the plates with iodine solution. The strains that yielded a high level of α -amylase were selected for further experiments. Microbiology of the isolated strains was determined according to the methods described in Bergey's Manual of Systematic Bacteriology (Sneath et al., 1986).

Media composition and cultivation conditions

Pre-inoculum cultures of selected strains were grown

in a 100 ml shake flask containing 15 ml LB media (composed of (g/l): tryptone, 10.0; yeast extract, 5.0; NaCl, 10.0.) and incubated overnight at 37°C in a rotary shaker at 150 rpm. Ground triticale grains (16g) were mixed with hot water (16 ml) and then incubated at 60°C for 3 h. The substrate was autoclaved at 121°C for 30 min. For solid-state fermentations, 2 ml of pre-inoculum cultures was transferred aseptically to a 300 ml shake flask containing previously prepared substrate. The flasks were incubated at 28 and 37°C, RH 50%, under static condition for 6 days.

Extraction of α-amylase

Fermented substrate was mixed thoroughly with 0.05 M phosphate buffer (pH 6.5) (1:5, w/v) and homogenized with an Ika Turrax homogenizer for 3 min with pauses. The slurry was mixed for 60 min at room temperature and then the whole content was centrifuged at 10 000×g for 10 min. The clear supernatants used before as the crude amylase extracts were desalting on Sephadex G25.

α-Amylase activity assay

Alpha-amylase activity was assayed by adding 0.05 ml of appropriately diluted enzyme to 0.45 ml soluble starch (1%, w:v) in 0.05 M phosphate buffer, pH 6.5, for 30 min at 55°C. The reaction was stopped and the reducing sugars determined with dinitrosalicylic acid according to the method of Bernfeld (1955). One enzyme unit is defined as the amount of enzyme that releases 1 μmol of reducing end groups per minute at the defined reaction conditions. D-maltose was used to construct a standard curve.

Zymogram of α-amylase

Isoelectric focusing was performed using the Multiphor II electrophoresis system (Pharmacia-LKB Biotechnology) according to manufacturer's instruction. Focusing was carried out on 7.5 % acrylamide gel with ampholytes in a pH range 3.0-10.0, at 7 W constant powers for 1.5 h at 10°C. After the run, α-amylases were detected by in-gel activity staining with I₂/KI staining solution according to a previously published

method (Dojnov et al., 2008). Alpha-amylase activity appeared as clear bands on a dark background.

Raw starch hydrolysis

The raw cornstarch digestion ability of crude α-amylase extracts was investigated by measuring the hydrolysis of 1% raw-starch granules after 24 h of hydrolysis at 65°C. Raw-starch degradation was monitored as previously described using the DNS acid method, with maltose as the standard (Božić et al., 2011). After 24 h of hydrolysis, starch grains were examined under light microscopy using a Leitz Diaplan microscope, at 400 times magnification. Pictures were taken using a digital CCD Nikon camera.

RESULTS AND DISCUSSION

Isolation and identification of microorganism

Seven of all tested strains on starch agar plates were selected on the basis of clear zones around the colony after 24 h of incubation (Fig 1). These isolates (2B, 4, 5B, 6, 18, 24B and 25A) were gram-positive, rod-shaped aerobic, catalase-positive and spore-forming. On the basis of various morphological and biochemical characteristics, they were identified as *Bacillus* sp. following the criteria laid down in Bergey's Manual of Systematic Bacteriology (Sneath et al., 1986). *Bacillus* sp. 24B appeared to be the most potent amylase-producing strain, since no starch was detected after reaction with the iodine solution (Fig. 1).

Effects of substrate and different growth temperature on α-amylase induction

In SSF, the selection of a suitable solid-state substrate is a critical factor (Tanyildizi et al., 2007). Inexpensive agriculture and agro-industrial residues are generally considered the best substrates for SSF processes and for enzyme production in these systems. Among the different substrates used for SSF, wheat bran has been reported to be a promising medium for α-amylase production (Nandakumar et al., 1996; Mulimani et al., 2000; Haq et al., 2003). Other substrates such as sunflower meal, rice husk, cottonseed meal, soybean

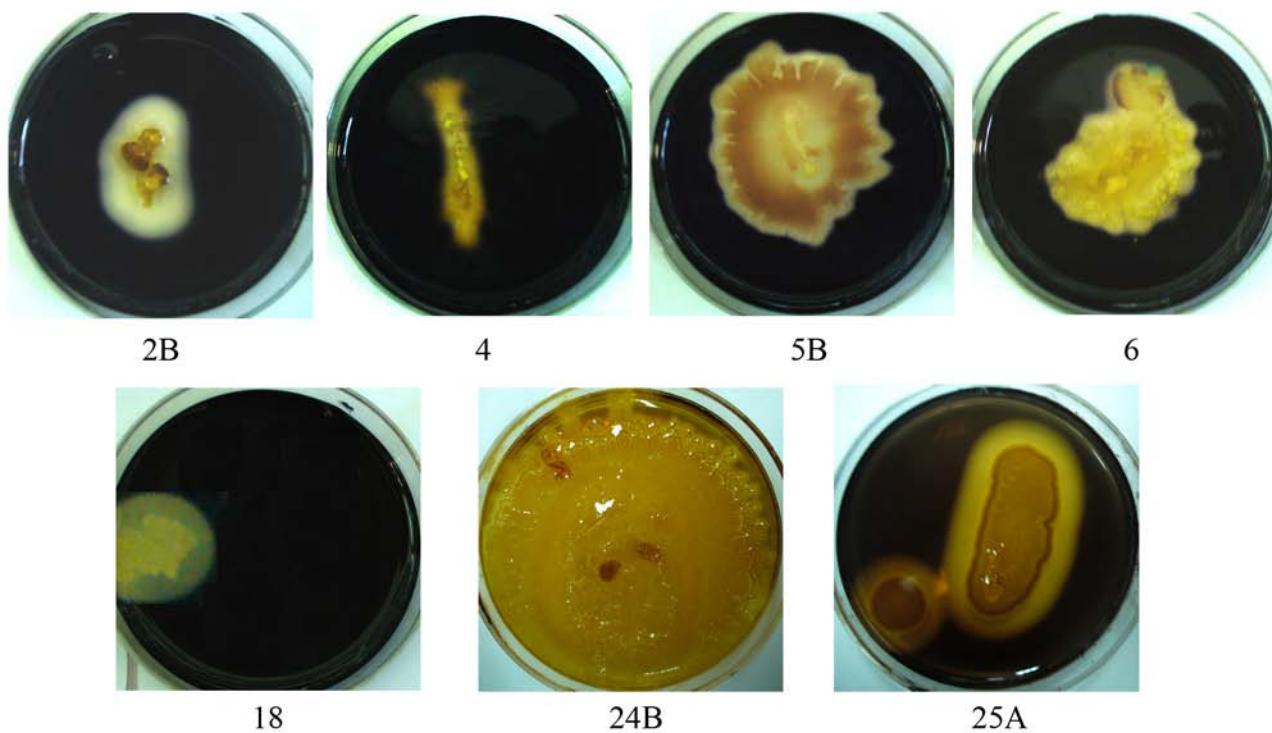


Fig. 1. *Bacillus* sp. isolates after starch agar plate assay with iodine for amylase producers.

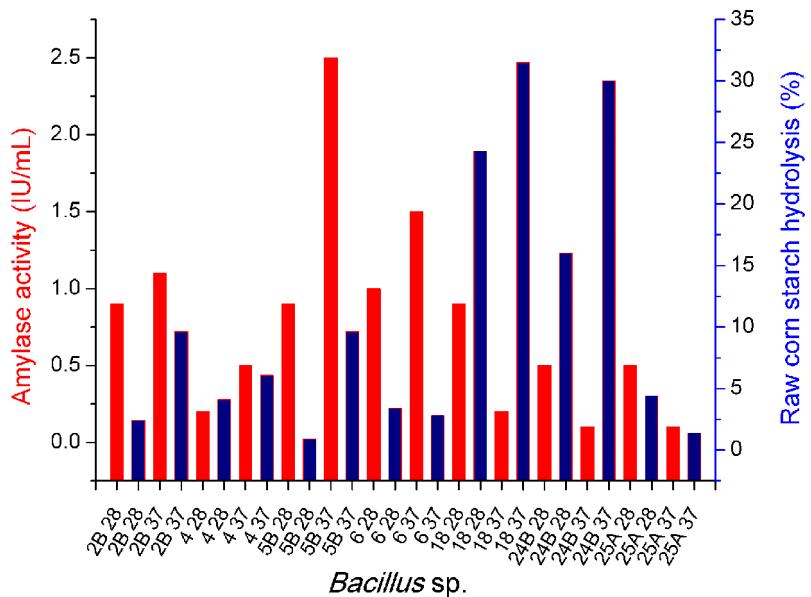


Fig. 2. Amylase activities in the extracts obtained after SSF of different *Bacillus* sp. strains at 28 and 37°C and corresponding hydrolysis of 1% raw cornstarch (RCSH) after 24 h of incubation.

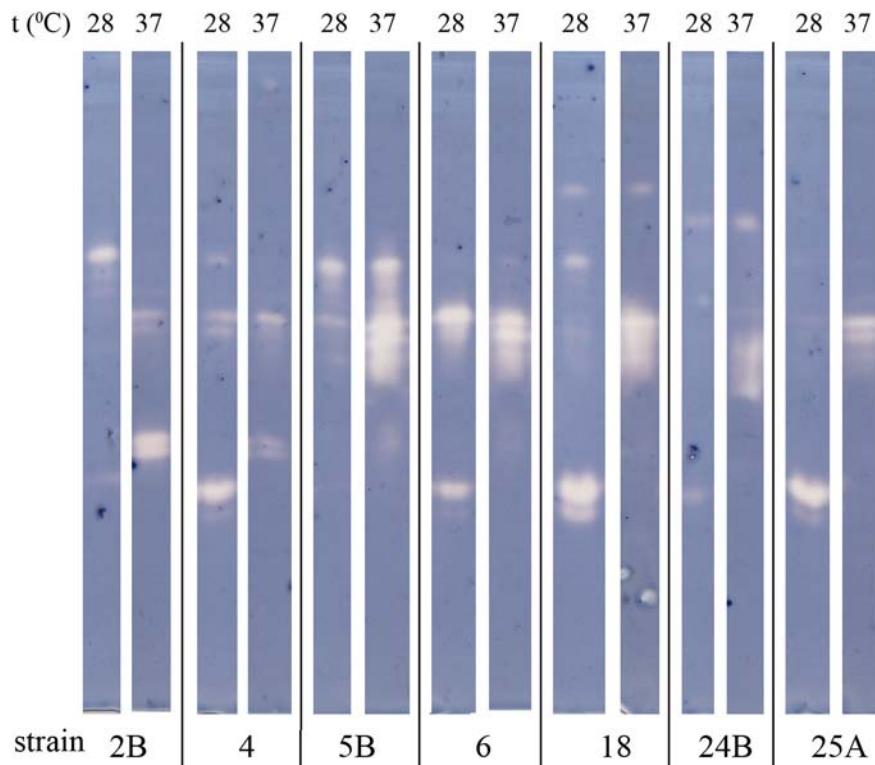


Fig. 3. Zymogram detection after isoelectrofocusing of amylase isoforms obtained after SSF of different *Bacillus* sp. strains at 28 and 37°C.

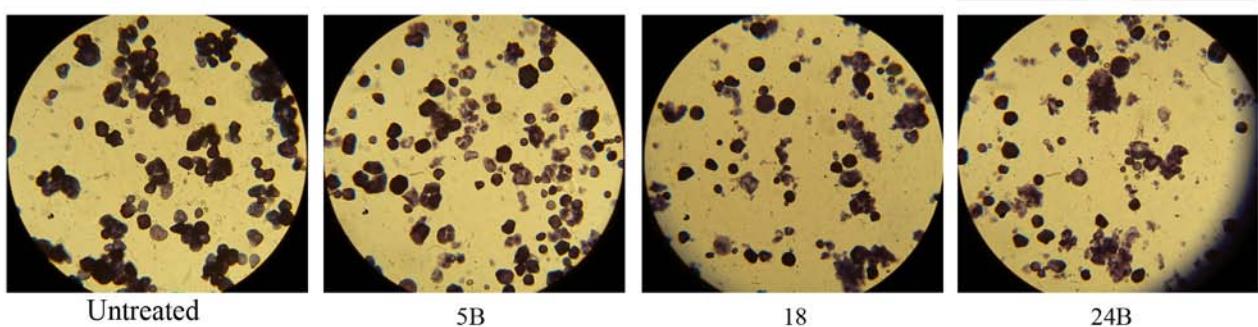


Fig. 4. Light microscopy of untreated and digested raw cornstarch granules after incubation with amylases from *Bacillus* sp. strains 5B, 18 and 24B.

meal, pearl millet and rice bran have been successfully used for SSF production of amylases (Haq et al., 2003; Baysal et al., 2003). We decided to test triticale (*Triticosecale*) grains, an important industrial crop, as a substrate for SSF because it is not sufficiently utilized yet. Cultivation of triticale has many benefits compared to other crops, such as high biomass and

grain yield over a wide range of soils (particularly resistant to low pH) and climate conditions, with yields from 3 to 6 t/ha depending on the geographic region (Salmon et al., 2004). Triticale contains high amounts of starch (about 60%), high amounts of proteins (from 12% to 15%), higher amounts of the major mineral elements (K, P, Mg) and nutritionally

important minor elements (Na, Mn, Fe, Cu, Zn) than wheat (Lorenz et al., 1974); Peña, 2004).

B. amyloliquefaciens, *B. subtilis*, *B. licheniformis* and *B. stearothermophilus* are among the most commonly used *Bacillus* sp. reported to produce α -amylase usually at temperatures 37-60 °C depending on the strain (Mielenz, 1983; Syu and Chen, 1997; Mishra et al., 2005; Mendu et al., 2005). However, in one study of α -amylase production by growing *Bacillus* sp. on SSF, the authors obtained a significant increase in enzyme production after lowering the incubation temperature from 45 to 28°C (Hashemi et al., 2010). Because of this, we decided to test the growth of different wild type *Bacillus* sp. strains at 28 and 37°C, as well as the production of different amylase isoforms. The results showed that enzyme production was maximal (2.56 IU/mL) after growing *Bacillus* sp. 5B at 37°C (Fig. 2). The production of α -amylases was different for all strains tested at both temperatures. The activity of enzymes was lower at 28 °C for the *Bacillus* sp. strains 2B, 4, 5B and 6, while at 37°C lower amylase activity was obtained for the *Bacillus* sp. strains 18, 24 and 25A.

Zymogram of α -amylase isoforms

To test amylase isoform production, extracts were loaded onto an isoelectrofocusing gel and amylases were detected after zymography using soluble starch as a substrate. Amylase activities in crude preparations were detected in the multiple forms for all seven strains *Bacillus* sp. tested; interestingly, the number and intensity of each form differed, depending on the growth temperature (Fig. 3). Previous reports showed the existence of multiple isoforms of amylase produced by *Bacillus* sp., such as three extracellular amylase isoenzymes produced by *Bacillus* species B-3881 (Boyer and Ingle, 1972) or six extracellular amylase isoenzymes produced by *B. licheniformis* ATCC 9945a (Božić et al., 2011). However, so far, there are no published data related to the study of the raw-starch hydrolysis ability of specific *Bacillus* sp. amylase isoforms or comparative studies with other isoforms when strains were grown on different temperatures.

Raw starch degradation ability of α -amylase

Gelatinization of starch requires a high-energy input resulting in an increased production cost of starch-based products. Hydrolysis of raw starch below gelatinization temperatures has gained importance in view of energy costs, effective utilization of natural resources and viscosity problems (Goyal et al., 2005). The raw cornstarch hydrolysis was investigated by monitoring the extent of hydrolysis of 1% starch granules with crude α -amylase extract (Fig. 2).

Amylases from *Bacillus* sp. strains 2B, 5B and 24B hydrolyzed the raw cornstarch when strains were grown at 37°C. *Bacillus* sp. strains 2B and 5B hydrolyzed 10% of starch after 24 h of incubation, while strain 24B hydrolyzed 30% of starch under the same conditions. Amylases from *Bacillus* sp. strain 18 were also very efficient in raw-starch hydrolysis, regardless of growth temperature. After growing the strain at 28°C, the obtained amylases hydrolyzed 24% of starch, while after growing at 37°C, 30% of hydrolysis was obtained.

Enzymes capable of digesting raw cornstarch granules were monitored with the support of light microscopy (Fig.4). After 24 h of hydrolysis, the number of granules was reduced while the structure of visible starch granules was more or less damaged.

Due to the different reaction conditions used, comparison with other *Bacillus* sp. amylases was difficult. However, for example, hydrolysis yields in a period of 24 h of raw cornstarch at 40°C were 32% for the amylase from *Bacillus* sp. IMD 435 (Hamilton et al., 1999) while at 60°C, hydrolysis was 60% for the α -amylase from *Bacillus* sp. GRE1 (Haki et al., 2008). It is very important to point out that approximately 10-100 times less enzymes were applied in our study.

The present study shows that the tested *Bacillus* sp. strains produced α -amylases able to hydrolyze raw cornstarch at a temperature below the temperature of gelatinization. However, after careful examination of zymograms, comparison of existence and

intensity of isoforms in tested *Bacillus* sp. depending on the different growth temperatures, with emphasis on differences, and by comparison with raw corn-starch digesting ability, it was clear that the ability for hydrolysis was not directly related to the total enzyme activity, suggesting that only certain isoforms are involved in the hydrolysis. To test this hypothesis, purification and examination of specific amylase isoform capability to hydrolyze raw-starch needs to be performed.

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