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# SCREENING FOR STARCH-HYDROLYSING BACTERIA

RAZIEH YAZDANPARAST

*From the Institute of Biochemistry and Biophysics, Tehran University of Medical Sciences, Tehran, Islamic Republic of Iran.*

## ABSTRACT

Screening of 3000 soil samples collected from cities of four different provinces of Iran for starch-hydrolysing bacteria revealed that the nature is enriched with Streptomyces species capable of producing amylolytic enzymes. Among the bacterial isolates, one of the high starch-degrading strains was selected for further microbiological identification and also amylolytic enzyme(s) characterization. The purified isolate, Streptomyces species strain RY48, produces almost four times more amylolytic enzyme(s) than *Bacillus subtilis* (PTCC 1254) on agar plates. Based on the action pattern of its amylolytic enzymes on the boiled maize starch, the purified species possesses in the logphase an amylolytic activity which is different from the amylolytic activity of the stationary phase. The secreted amylolytic enzymes which are both endolytic type and show different activity bands on native polyacrylamide gel, act independently on starch molecules.

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**Keywords:** Amylolytic enzymes, amylases, raw-starch, Streptomyces.

## INTRODUCTION

Starch, as a major component of agricultural crops, is an important source for conversion into materials used mainly in foods, drinks, textiles, adhesives, and pharmaceutical industries. Enzymatic hydrolysis of polysaccharides such as starch and glycogen into oligosaccharides or simple sugar constituents is achieved by different amylolytic enzymes produced by a variety of microorganisms. Amylases (such as: ∝-amylase, ∝-1,4-D-glucan gluconohydrolase, EC 3.2.1.3.) are among the most biotechnologically applied amylolytic enzymes.[1,2]

These enzymes are isolated from a variety of microbial sources: ∝-amylase is produced mainly by *Bacillus subtilis*, *B. Amyloliquefaciens*, and *Aspergillus oryzae*; and glucoamylase is produced by Rhizopus species and *Asperigillus niger*.[3,6]

Regarding the fact that the enzymatic hydrolysis of native starch granules requires precooking of starch into a starch solution, the elimination of this energy-consuming step from the starch-processing steps is of great interest to starch-processing industries. Achievement of this goal is sought in the replacement of the presently used enzymes with (1) mixed amylolytic enzyme system(s), and /or (2) raw- starch hydrolysing enzymes purified from new microorganisms. The significance of the first approach can be implied from the simultaneous action of bacterial ∝-amylase and fungal glucoamylase on starch molecules. It has been indicated by Fajii and co-workers that the hydrolysis of starch molecules by combined action of ∝-amylase and glucoamylase is almost twice as much as the sum of the corresponding actions of sole ∝-amylase and sole glucoamylase systems.[7,8] This positive cooperativity between the two enzymes is explained to be due to the gradual substrate release for glucoamylase by the random endolytic splitting action of ∝-amylase on starch molecules. Despite the more efficient hydrolysis of starch molecules by the mixed-enzyme system, the industrial application of this method is limited due to the end-product inactivation of ∝-amylase and glucoamylase. Therefore, it appears that the elimination of energy-consuming step (precooking) in starch-processing industries could prob-



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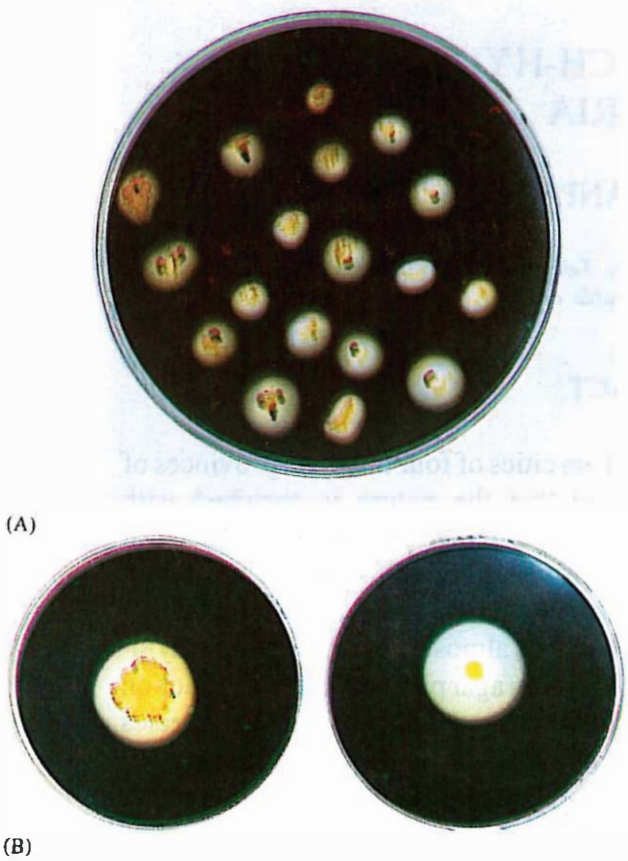


**Fig. 1.** Selective isolation of starch-hydrolysing bacteria by baiting technique. Bacterial Colonies showing clear zones after floating with Lugol's iodine solution are counted as starch-hydrolysing microorganisms, A; Clear zones formed by *B. subtilis* (a) and the purified strain RY48 (b), B.

ably be achieved in the future by using a single enzyme capable of hydrolysing raw-starch molecules or a mixed-enzyme system whose enzyme constituents are derived from a single microorganism.

Our main research goal was to investigate the nature of Iran for its bacteria capable of hydrolysing raw starch molecules by their corresponding amylolytic enzyme(s) and further to characterize these enzymes for further aim of cloning the corresponding genes and analysing their structure for design of an efficient expression system of the genes (or genetically engineered genes) in a suitable host-vector.

## MATERIALS AND METHODS

**Materials:** Bacterial ∝-amylase (695 units/mg solid), mold glucoamylase (6.3 units/mg solid), and maize starch were purchased from Sigma Chemical Co; acetone, dinitrosalicylic acid, propan-2-1, and lactic acid were from

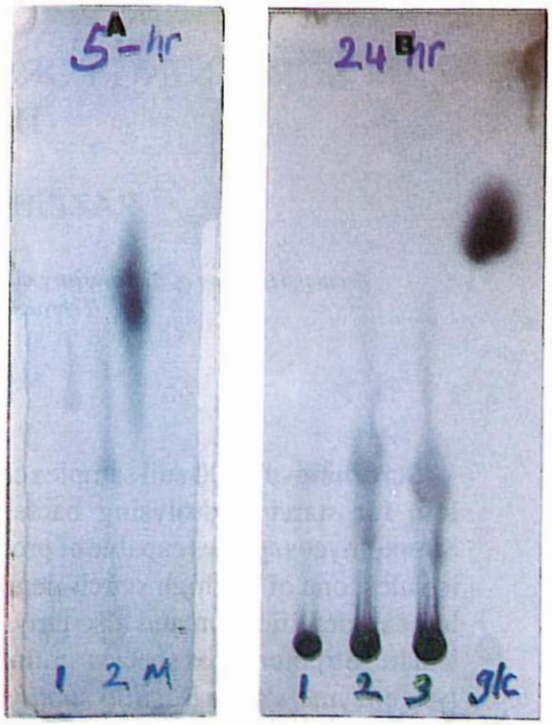


**Fig. 2.** Thin-layer chromatography of the products formed from starch solution in five hours (A) and twenty two hours (B). The action pattern of enzyme(s) present in 48-hour, 1; 120-hour, 2; and combined, 3; culture supernatants are shown along with reference compounds: 0.1% maltose (M) and 0.1% glucose (glc).

Aldrich. Silica gel plates (0.25 mm) were obtained from MacHerey-Nagel Co, nutrient broth and agar were from Difco, and polyacrylamide, N,N-bisacrylamide, TEMED, and ammonium persulfate were purchased from Pharmacia /LKB.

All experiments were performed in glassware, and distilled water with conductivity of 4 to 5 was applied throughout the investigation. All materials were applied for the investigation without further purifications.

**Microorganisms:** All bacteria were isolated from soil samples which were collected from different areas of Tehran, Karaj, Rasht, Ahvaz, and Babolsar.

**Media:** The media used for the screeing were Medium A (g/l): nutrient agar, 30; maize starch, 2; agar, 15; and medium B (g/l): nutrient agar, 30; agar, 15. After autoclaving to about 40°C, autoclaved raw maize starch, 2, was added to the solution. Enzyme production Medium (g/l) consisted of nutrient broth, 16; maize starch, 2.

**Screening: Method 1.** One gram of each moist soil sample was suspended in 3 ml sterile water, mixed, and then streaked onto the plates of Medium A described above. The plates were incubated at 37°C for 24 to 76 hours. Bacteria that formed large clear zones, in comparison to the corresponding zone produced by *Bacillus subtilis*



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(PTCC 1254) were further purified by plating on the same plates until purified single colonies were obtained. Each of the selected isolates were then spotted onto agar plates of Medium B. The plates were incubated in a moist incubator at 37°C for 120 hours. The bacteria which formed clear zones of 2 mm or more around their corresponding colonies were selected for identification and further studies.

**Method 2.** We used the method described by Umesh-Kumar[9] with minor modifications for selective screening of soil samples. Raw, healthy pealed potato discs were buried in very moist soil samples and kept at room temperature (27-28•C). After 48 hours of baiting, the discs were superificially washed with sterile water and kept in sterilized petri dishes in an incubator for 48 hours at 37°C. Potato discs were then ground in sterile water and serial dilutions of each potato disc were plated onto agar of Medium A and kept in an incubator at 37°C. Furtherprocesses of purification were the same as described in method 1.

**Enzyme Production.** The purified microorganism of interest was grown in 10 mL of enzyme production medium for 24 hours at which was found to be the best temperature for growth. 2 mL of the inoculate was then transferred to 100 mL of the saune medium and kept at 37°C up to 120 hours while shaking at 90 rpm. The broth was then centrifuged at 3000 rpm for 20 min at 4°C. The supernatant was used to determine the total protein content and to assay for amylolytic enzyme activities.

**Protein Determination.** Protein content of the culture supernatant was assayed using the method of Lowry[10] with bovine serum albumin as standard.

**Enzyme Activity.** The reaction mixture consisting of 400 µL of a 2.5% (W/V) maize starch solution in acetate buffer (0.05 M sodium acetate + 0.05 M acetic acid, pH 5.3) and 200 µL of the culture supernatant plus 1400 µL acetate buffer, was incubated at 37°C for up to 6 hours and /or overnight. Samples were taken at different time intervals. After removal of residual starch granules by centrifugation at 3000 rmp for 10 min, the reducing sugar liberated was determined by the dinitrosalicylicacid method with maltose as the standard. [11] One unit of saccharifying activity was defined as the amount of enzyme which liberated 1 mg of reducing suger in 1 hr under the assay conditions. After 6 hours, the remaining reaction mixture was centrifuged and the supernatant was boiled for 5 min to deactivate the enzymes and kept at 4°C for thin layer chromatography examination of the products.

**Thin Layer Chromatography (TLC).** 5 µL of each sample was spotted on silica gel plates and developed in the following solvent system: propan-2-01: acetone: 1M lactic acid (4:4:2). After completion of chromatography, the plates were sprayed with a reagent consisting of aniline (4 mL), diphenylamine (4 g), acetone (200 mL) and 85% $H_3PO_4$ products appeared as blue spots on the white background of the silica gel plates.

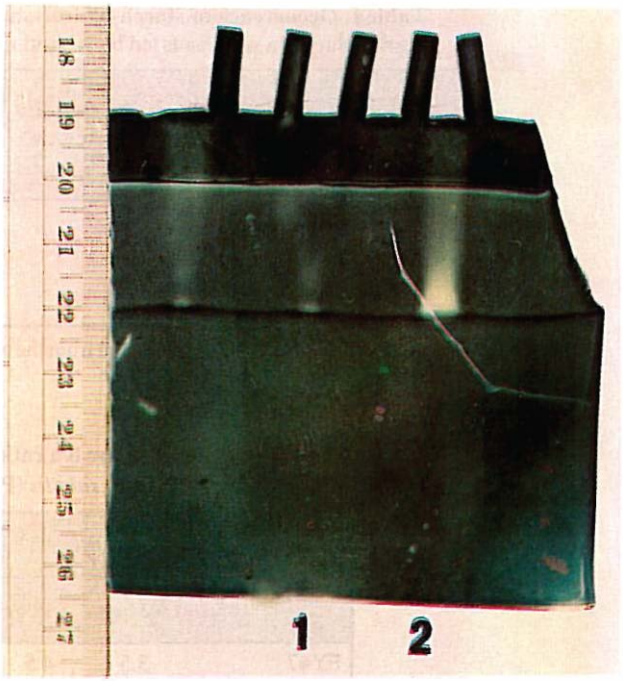


**Fig. 3.** Native polyacrylamide gel electrophoresis of the 48-hour (Lane 1) and 120-hour (Lane 2) culture supernatants of the purified microorganism, strain RY48. Non-denatured electrophorsis was achieved according to Lacks and Springhorn's technique.[13] Gel thickness, 1.5 mm.

**Gel Electrophoresis.** Discontinuous native polyacrylamide gel electrophoresis was achieved at 50 mA constant current according to the method described by Laks and Springhorn.[13] The gel thickness was 1.5 mm. The enzyme bands were detected after electrophoresis using boiled soluble maize starch as described in reference 13.

**Semi-Identification of the Bacterial Isolates.** Cultural, morphological, and some of the biochemical characterizations of the purified microorganisms were performed according to Capuccino and Shermans Manual.[14] *Bacillus subtilis* (PTCC 1254) and *Streptomyces rimosus* (PTCC 1143) were used as references. The results were analysed using "Bergey's Manual of Systematic Bacteriology."[15]

## RESULTS

### Screening for Starch Hydrolysing Bacteria

Results of burying potato discs in low-depth soil samples to bait for starch-hydrolysing bacteria indicated the abundance of these microorganisms in top layers of soil. As shown in Table 1, baiting of soil samples with potato discs as a nutrient assisted the enrichment of starch-hydrolysing bacteria on the potato discs and consequently their selective isolation from soil in comparison to streaking method of screening. As indicated in Fig. 1, almost all microbial



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**Table I. Occurrence of starch-hydrolysing bacteria in four soil samples* collected from Karaj. Bacteria were isolated by streaking (method 1) and baiting (method 2) techniques.**

| Sample | %Starch-hydrolysing bacteria isolated by baiting technique | %Starch hydrolysing bacteria isolated by streaking technique |
|---|---|---|
| 1 | 100 | 10 |
| 2 | 95 | 12 |
| 3 | 98 | 9 |
| 4 | 98 | 20 |

* The soil samples have been collected from the top layer ground with a depth of less than 5 centimeters.

**Table II. Comparison of the b/a ratios of the purified amylolytic isolates to the corresponding ratio of *B. subtilis* (PTCC 1254) in a time course investigation.**

| Strain No. | b/a | | | | |
|---|---|---|---|---|---|
| | 24 hr | 48 hr | 72 hr | 96 hr | 120 hr |
| FY47 | 3.5 | 4.5 | 5.3 | 6.3 | |
| RY48 | 4.0 | 4.3 | 4.0 | 3.9 | |
| RYT6 | 2.7 | 3.5 | 3.6 | 3.7 | |
| RY34 | 2.7 | 3.3 | 4.8 | 3.6 | |
| *B. subtilis* | 1.4 | 1.4 | 1.2 | 1.1 | |

* b/a = b, the diameter of the clear zone; a, the diameter of the bacterial colony, both quantities in centimeters.

colonies indicated clear zones around their corresponding colonies after adding Lugol's iodine solution on the plates. Using the selective method of baiting, a total of 3000 soil samples were screened primarily for starch-hydrolysing bacteria. Microorganisms which possesed the largest b/a ratio [ratio of clear zone diameter (b) to colony diameter (a)] were picked up for further investigation. Using this criteria, and *Bacillus subtilis* (PTCC 1254) as the reference and under all identical growth conditions, five microorganisms were selected with b/a ratio of more than twice the b/a ratio of *B. subtilis* in the first 96 hours of growth (Table II). The selected microbial species were then spotted on agar plates of Medium B which contained raw maize starch granules and kept at 37°C in a low-moist incubator for 96 to 120 hours. Using Lugol's iodine solution, it was shown that all the purified isolates are capable of producing a clear zone around their corresponding colonies (data not shown). However, the diameter of the clear zones were from 1 to 3 mm larger than the corresponding colonies' diameters. In addition, the clear zones became apparent after almost 48 hours of growth. This observation is in contrast to the appearance of the clear zone around the colonies on agar plates of Medium A which usually appeared in the first 10 to 15 hours of growth. One of the isolates, strain No. RY48, which grows faster than the strain RY47 (Table II) was selected for further studies.

**Characterization of the Isolated Microorganism, Strain RY48**

Some of the characteristics of strain RY48 are shown in Table III. However, chemotaxonomical studies with the determination of DNA base compositions, which were not performed in the present work, are very necessary for definitive identification of this microorganism. Based on the phenotypic properties, the isolated microorganism belongs to the genus Streptomycetes. Although definitive species identification has not been done yet, tentatively, we named the purified microorganism Streptomyces sp. strain RY48.

**Properties of Amylolytic Enzyme(s) Produced by Strain RY48**

The action pattern of amylase(s), produced by strain RY48, on maize starch solution showed that the isolated strain possesses at least two kinds of amylolytic activities. As shown in Fig. 2, in the first 48 hours of growth an enzyme is produced which hydrolyses starch molecules into oligasaccharides different from the major product(s) formed from the 120-hour of the hydrolysates, the product of the latter enzymatic activity has higher molecular weights than the product(s) of the former enzymic activity. Time-dependent hydrolysis of starch molecules by 48 and 120-hour culture supernatants showed that the action pattern of



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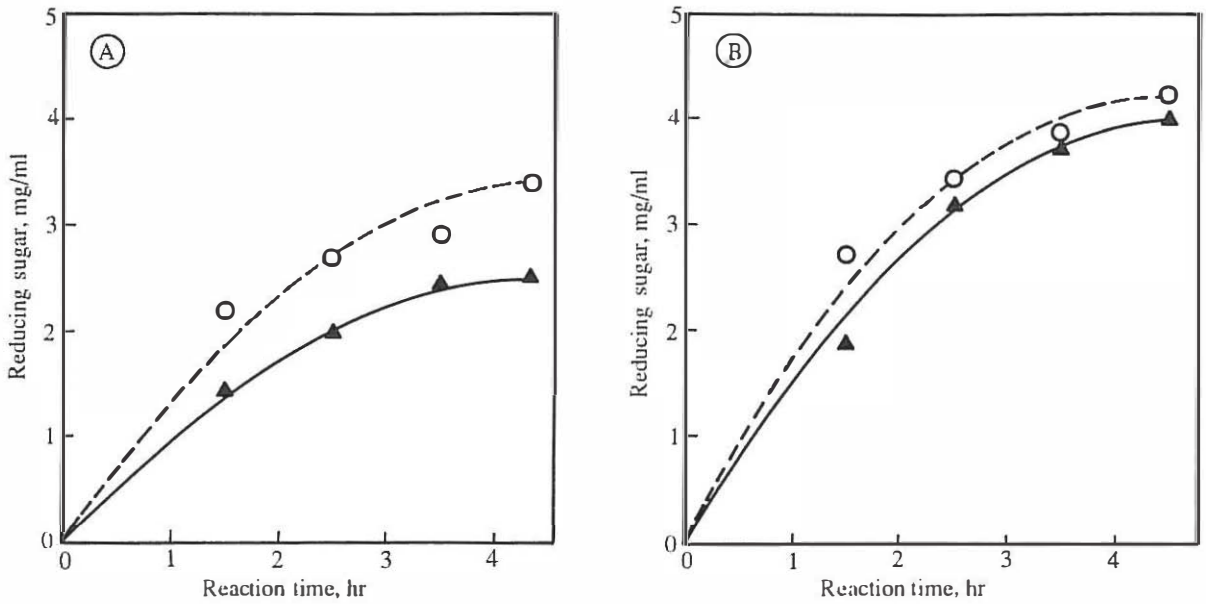


**Fig. 4.** Reducing sugar formation by the mixed enzyme system of α-amylase (26 u/mL) and glucoamylase (0.1 u/mL) in time course experiments at a starch concentration of 5 mg/mL (A) and 10 mg/mL (B). Solid line = observed data; broken line = calculated data.

both kinds of amylolytic activities remained unchanged during 22 hours of reaction and no significant amount of monosaccharides were detected in the reaction mixtures using TLC technique. These observations indicate that both amylolytic activities are of endolytic type. It is interesting to note the presence of two different clear zones on the agar plate too (Fig. 1b). This observation may support the existance of at least two kinds of hydrolytic activities on starch molecules. Native polyacrylamide gel electrophoresis of the 48- and 120-hour culture supernatants followed by assaying for amylolytic activities indicated different activity bands. As shown in Fig. 3, the 120-hour culture supernatant is mainly enriched of labeled a as compared to the enzymes of the 48-hour-culture superna-

**Table III. Phenotypic Charaterization of Strain RY48**

| | |
|---|---|
| **Cultural Characteristics** | |
| Nutrient agar colonies: | flat, yellowish, slightly more than moderate growth, wet |
| Nutrient agar slant : | flat, yellowish, moderate growth, wet |
| Sabouraud-dextrose agar colonies : | No growth |
| Nutrient broth : | light turbidity, moderate growth |
| **Morphological Characteristics** | |
| Mycelium forming on agar plate with lots of branches, fragile mycelium, broken easily into irregular-size rods with slight agitation, non-spore forming, non-acid fast, Gram-negative. | |
| **Biochemical Characteristics** | |
| VP - test | - |
| MR - test | - |
| Starch hydrolysis | + |
| Casein hydrolysis | - |
| Nitrate to Nitrite conversion | + |
| Acid formation from glucose | + |
| Acid formation from maltose | + |
| Acid formation from galactose | + |

* VP test= Voges-Proskauer test

** MR test= Methyl Red test



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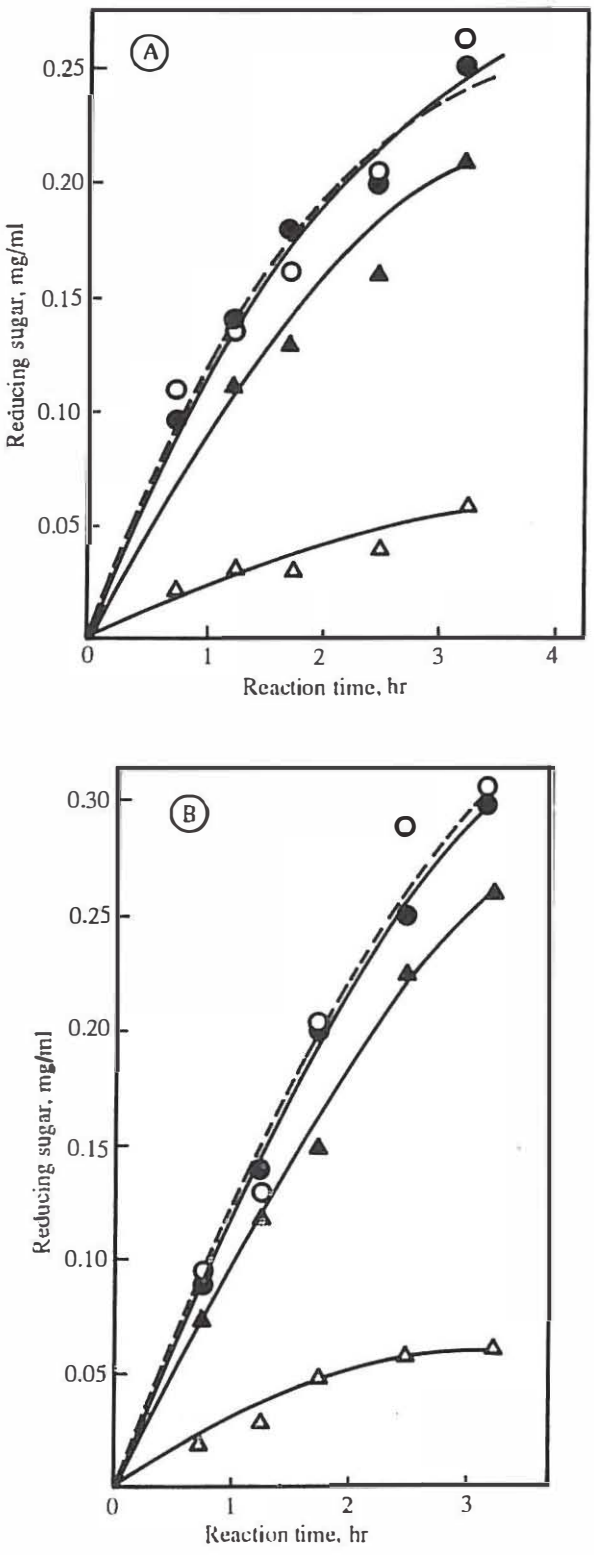


**Fig. 5.** Reducing sugar formation by the culture supernatants of 48-hour (△——△), 120-hour (▲——▲), and combined supernatants (●——●) in time-course experiments at a starch concentration of 5 mg/mL (A) and 10 mg/mL (B); calculated data (○——○).

tant (bands a and b). These data further support the fact that there are at least two kinds of amylolytic enzymes responsible for starch degradation.

### Cooperative Actions of Amylolytic Enzymes of Strain RY48

Although detailed kinetic investigations of the amylolytic enzymes produced by the purified strain RY48 awaits complete purification of the enzymes, we attempted to get some knowledge about the possible cooperativity which may exist between the catalytic action of the enzymes in hydrolysing starch molecules. The hydrolysis of starch molecules by *Bacillus subtilis* α-amylase and fungal glucoamylase were used as references. As shown in Fig. 4, the mixed enzyme system of α-amylase and glucoamylase at starch concentration of 5 and 10 mg/ mL produced 36% and 11% more reducing sugars, respectively, than the sum of the sole action of α-amylase and glucoamylase on starch solution under all identical experimental conditions. Despite this observation, our data does not support such a cooperativity (either positive or negative) between the amylolytic enzymes of strain RY48. As shown in Fig. 5, there is almost no difference in the amount of reducing sugar formed by the mixed enzyme system (mixed 48- and 120- hour culture supernatants) and the sum of the individual enzyme solutions, under all identical conditions, at starch concentrations of 5 and 10 ml/mL. These observations may preserve independent hydrolytic roles for the amylolytic enzymes of the purified strain RY48.

## DISCUSSION

On screening the top layer soil samples collected from different regions of Iran, it became clear that high-producing bacterial strains capable of hydrolysing starch molecules are widely distributed in nature. The high success in isolation of starch hydrolysing microorganisms from soil samples is largely dependent on the screening techniques applied for purification. As shown in Table I, baiting of top layer soil with raw potato discs is an efficient technique for enrichment of soil samples with interested microorganisms before the process of purification, in comparison to streaking techniques in which purifications are achieved without enrichment of starch-hydrolysing microorganisms. The purified starch-degrading bacteria were also capable of hydrolysing raw starch granules of their medium. However, this activity was not very strong and usually appeared after the second day of growth on agar plates. The best isolate in this regard was found to be a Streptomyces which was tentatively named streptomyces Sp. strain 48 and was subjected to further investigation regarding the properties of its amylolytic enzymes.

The action pattern of the amylolytic enzymes produced hy streptomyces strain RY48 on soluble starch solution



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suggests that the excreted enzymes showed mainly two kinds of activities in production of oligosaccharides from starch molecules. As shown in Fig. 2, the activity associated with the 48-hour culture supernatant degrades the starch molecules into oligosaccharides with molecular weights smaller than the molecular weights of the oligosaccharides produced by the amylolytic activity associated with the 120-hour culture supernatant. In addition, it should be mentioned that the enzymic hydrolysates of both kinds of culture supernatants did not contain simple sugars such as glucose and maltose as shown by TLC technique (Fig. 2). These observations suggest that the purified strain produces mainly endolytic amylolytic enzymes which enable the microorganism to degrade starch molecules into oligosaccharides. However, as shown in Fig. 5, the amylolytic activities associated with the culture supernatant of strain RY48 degrade starch molecules independent of each other. In other words, our data did not support the existence of positive cooperativity between the two different amylolytic activities, in comparison to the positive cooperativity which exists between the mixed-enzyme system of α-amylase and glucoamylase, as shown in Fig. 4. Further investigation regarding the physical and chemical properties of these enzymes awaits complete purification of the enzymes. These investigations are in progress and will be described in subsequent reports.

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