hiop. J. Biol. Sci., 4(2): 147-160, 2005
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THERMOSTABLE, RAW-STARCH DIGESTING α-AMYLASE FROM *BACILLUS STEAROTHERMOPHILUS* GRE 1

Gulelat Desse*[1] and S.K. Rakshit[2]

ABSTRACT: A thermophilic bacterium, *Bacillus stearothermophilus* GRE 1 isolated from an Ethiopian hyperthermal spring produced raw starch digesting thermostable α-amylase. Enzyme production in shake flask studies (using optimum nutrient supplements and environmental conditions) reached 2.36 U/ml. Gel filtration chromatography yielded a purification factor of 33.62-fold and a recovery of 46.52%. The optimum temperature for activity was determined to be 60-70°C and optimum pH was in the range of 5.5-6.0. The enzyme maintained 50% of its original activity after 45 minutes of incubation at 80°C and is stable at pH values of 5.0-9.0. Cu^{2+}, Zn^{2+} and Fe^{2+} strongly inhibited enzyme activity. The enzyme is calcium-independent and 94% and 86% relative activity were displayed with low concentrations of Co^{2+} and Mg^{2+}, respectively. The enzyme hydrolyzed wheat, corn and tapioca starch granules efficiently.

Key words: *B. stearothermophilus*, Hyperthermal spring, Raw starch digesting, Thermostable α-amylase

INTRODUCTION

One of the most prominent and captivating aspects of the microbial world is its rich structural and functional diversity. Understanding the span of its miscellany is constantly intensifying as formerly unidentified species are revealed, studied and novel metabolic processes are elucidated. Advances in the area have been possible with the isolation and screening of beneficial extremophiles from exotic regions, which are capable of producing enzymes active at high temperatures.

Every year 2^{10} tons of starch is produced by land plants, which provides four-fifth of the world's food, calculated as calories (Sarikaya *et al.*, 2000) and the most widely used thermostable enzymes are the amylases in the starch industry (Lèvèque *et al.*, 2000). Although thermostable enzymes have found a number of commercial applications because of their overall inherent stability (Gulelat Desse and Rakshit, 2003), screening of a strain capable of producing commercially acceptable yields of amylases has remained to be a

*1 Department of Food Science and post harvest Technology, Hawassa University, PO Box 1703, Awassa, Ethiopia. Tel +251 0916 823262, Fax +251 046 221 47 81, gulelatw@yahoo.com
[2] School of Environment, Resources and Development, Food Engineering and Bioprocess Technology Program, Asian Institute of Technology, P.O.Box 4, Klong Luang, Pathumthani 12120, Thailand

challenging task (Pandey *et al.*, 2000). Key to the industrial saccharification of starch used in syrup production, brewing, textiles, papermaking etc is gelatinization, achieved by heating starch with water and thereby enhancing its bioavailability to amylolytic enzymes.

Presently, specific thermostable amylolytic enzymes are of considerable biotechnological interest as their enhanced stability could greatly reduce enzyme replacement costs and permit processes to be carried out at high temperatures. In addition, since starch appears to be the only substrate in nature hydrolyzed by amylases, isolation of efficient strains capable of producing enzymes active on raw starch is enviable (Pandey *et al.*, 2000). Therefore, characterization of amylolytic enzymes of new strains continues to be of importance (Lèvèque *et al.*, 2000) and programs to select new microorganisms for enzyme production are increasing around the world (Stamford *et al.*, 2001).

The objective of this paper was to describe the isolation and identification of a strain capable of producing a thermostable and raw starch digesting α-amylase and characterization of the enzyme.

MATERIALS AND METHODS

The Great East African Rift (GEAR) is famous world-wide for its active rifting and volcanism in sub-aerial conditions from developed to early rifting stages. The hot spring from which the organism was isolated is located at Shalla and Abijata and lies within the Ethiopian rift system which involves the northern part of GEAR. The altitude of the area is 1560 meters above sea level and the temperature and pH of the spring, respectively, were 93°C and 8.

Screening, isolation and identification of the microbial strain

Water and soil samples were collected between January-August, 2002. A steel pipe with a diameter of 50 mm was used to take soil samples. The pipe was pressed 50-100 mm down into the soil to take the sample. Samples were then deposited in sterile bottles with a screw lid. After each sampling the pipe was cleaned thoroughly in 70% alcohol. At each position five soil samples were taken which were randomly distributed over an area of $1m^2$. Water samples from the hot springs were taken directly with a two meter long plastic pipe pressed 10-20 cm into the bottom. Temperature and pH of the water were also measured.

Enrichment of well-mixed soil and water samples was accomplished using nutrient broth supplemented with 0.5% (w/v) starch (Merck) and incubating

at 65°C overnight. A sample of 10 g was then diluted in 90 ml of distilled and sterilized water. Replicate starch agar plates were then spread-plated with 0.1ml of the diluted samples and incubated at 55°C for 12 to 24 h after which they were stained with Gram's iodine solution (0.1% I_2 and 1% KI) Colonies with the largest halo-forming zones were isolated for further scrutiny. Identification of the selected strain was conducted at the Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, according to Analytical Profiles Index (API) 50CHB test (Bio-Mérieux).

Enzyme production

The optimum nutrient supplementation for enzyme production was used as the final basal medium (Gulelat Desse, 2003). One hundred milliliters medium in 500 ml baffled flasks were inoculated with 4 ml of an overnight bacterial culture. Cell-free culture supernatant obtained after separating the cells by centrifugation (10000g for 15 minutes) was used for partial purification.

Enzyme assay

α-amylase activity was monitored using 3,5-dinitrosalicylic acid reagent (Bernfeld, 1955). A volume of 0.5 ml of enzyme solution (culture supernatant) was added to 0.5 ml of soluble starch solution (1% w/v) in 0.02 M sodium phosphate buffer, pH 6.9 and incubated for 3 minutes at 25°C. One ml of 3,5-dinitrosalicylic acid reagent was added and tubes were placed in a boiling water bath for 15 minutes after which they were cooled at room temperature and 10 ml of distilled water was added to stop the reaction. Absorbance at 540 nm was measured using a Unicam UV2 model spectrophotometer. One α-amylase activity unit was defined as the amount of enzyme that produced one micromole of maltose per minute under the assay condition.

Partial purification of amylase

Culture supernatant was brought to 75% ammonium sulfate saturation and the precipitate recovered was dissolved in a minimum volume of 20mM phosphate buffer, pH 6.0. The solution was further dialyzed against the same buffer at 4°C overnight followed by gel filtration chromatography, carried out using Sephadex G-100 column equilibrated with 20 mM phosphate buffer, pH 7. Amylase activity of each fraction was measured. The A_{280} was also used for monitoring protein in column effluents. Active fractions were pulled and used as the partially purified enzyme preparation.

Effects of pH and temperature on activity and stability

The optimum temperature for the activity was determined in 0.02 M phosphate buffer, pH 6.9 by varying the temperature of the reaction system (40-100°C) and the optimum pH was determined by conducting enzyme assay at 60°C (at the optimum temperature) using different buffers (acetate (4.0-6.0), imidazole (6.5-7.5), Tris-HCl (8.0-8.5) and glycine NaOH (9.0-10)), all at a concentration level of 50mM.

Thermostability assays were performed by incubating enzyme solution at 60-100°C in 20mM sodium phosphate buffer, pH 6.0. The enzyme was kept at 4°C for 30 minutes using buffers as above to determine pH stability. The remaining activity was measured at 60°C.

Effect of metal ions on enzyme activity

Enzyme was incubated with chloride salts of Na^+, Ca^{2+}, Co^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+} at 60°C in 20 mM sodium phosphate buffer, pH 6.0. The activity of the enzyme assayed in the absence of cation was taken as 100%.

Hydrolysis of raw starch granules

Twenty five mg of raw tapoica, corn, wheat and potato starch granules in 15 ml centrifuge tubes were hydrolyzed using 10 U/ml of the partially purified amylase in 0.625 ml of 20 mM phosphate buffer pH 6 and 1.625 ml of water. Toluene (0.05 ml) was added to prevent bacterial growth and samples were incubated at 40, 50 and 60°C in a shaking water bath (150 rpm). Samples were collected at regular time intervals (6, 12, 18, 24 and 36 hrs) and the production of reducing sugars was estimated by the method of Bernfeld (1955). Raw starch digestion rate (r_d) was defined according to the following equation (Iefugi *et al.*, 1996):

$$r_d(\%) = (A_1/A_0) \times 100$$

Where A_1 = wt of reducing sugar obtained from calibration curve for maltose A_0 = wt of whole raw starch before the reaction

Morphological changes in starch granules after digestion were monitored by a Scanning Electron Microscope (SEM). Starch granules, both native and digested, were washed with ethanol, dried and attached to an SEM stub with a silver plate. The mounted samples were coated with gold/platinum before observation.

RESULTS AND DISCUSSION

Enzyme production and identification of strain

The thermophilic strain previously designated as GRE 1 (Gulelat Desse, 2003) was later identified and named as *Bacillus stearothermophilus* GRE 1. The optimum growth temperature of the organism on starch agar plate was found to be 55°C. In a liquid media the organism was able to grow well at 60°C.

Over-production of primary or secondary metabolites is complex (Amartey *et al.*, 1991), and requires mastery of the fermentation process, media optimization and the fine-tuning of process conditions for each new strain. On top of that, aerobic or anaerobic conditions may affect the nutritional requirements of a bacterium (Lee *et al.*, 1991). The identification of the essential growth requirements has traditionally been done in shake flasks by adding or omitting certain nutrients and incubating statically or shaken. In the present study, the effect of the commonly used carbohydrate sources (starch, lactose, glucose, maltose), nitrogen sources (yeast extract, beef extract, peptone, ammonium sulphate), temperature, initial pH, degree of aeration and metal ions (Ca^{2+}, Mg^{2+}, Cu^{2+} and Zn^{2+}) on the enzyme production of *B. stearothermophilus* GRE 1 was studied in an attempt to develop better media and fermentation conditions in shake flasks. Accordingly, the final medium developed contained starch, 1 (w/v); lactose, 2.5 (w/v); yeast extract, 0.5 (w/v); NaCl, 1g; K_2HPO_4, 2g; $CaCl_2$, 0.1g and $MgSO_4$, 0.1g in one liter of distilled water with an incubation water bath temperature of 55°C, neutral initial pH and 200 rpm. While using this medium under the conditions stated, *B. stearothermophilus* GRE 1 yielded 2.36 U/ml of amylase, which was approximately 1.31-fold higher than the yield obtained from the original medium after 24 h of fermentation.

In similar studies conducted using *Bacillus* sp. IMD 435, an amylase activity of 26 U/ml was obtained using 4% lactose in the enzyme production medium (Hamilton *et al.*, 1999). According to some reports, thermostable amylolytic enzyme production from a *Clostridium* isolate and *Bacillus stearothermophilus* did not increase by increasing starch concentration (Madi *et al.*, 1987; Wind *et al.*, 1994). The possible explanation, which was further indicated by the authors, was the repression of enzyme synthesis at higher concentrations of starch-hydrolysis products such as glucose or a difference in O_2 availability due to a change in viscosity at higher starch concentrations or a combination of both.

Purification of α-amylase

As depicted in Figure 1, it was possible to partially separate the enzyme by gel filtration chromatography using Sephadex G-100 in which fractions 19-28 showed a higher pick of enzyme activity. The elution fractions were pooled and used for further characterization. Purification factor was 33.62 fold with a recovery of 46.52% (Table 1).

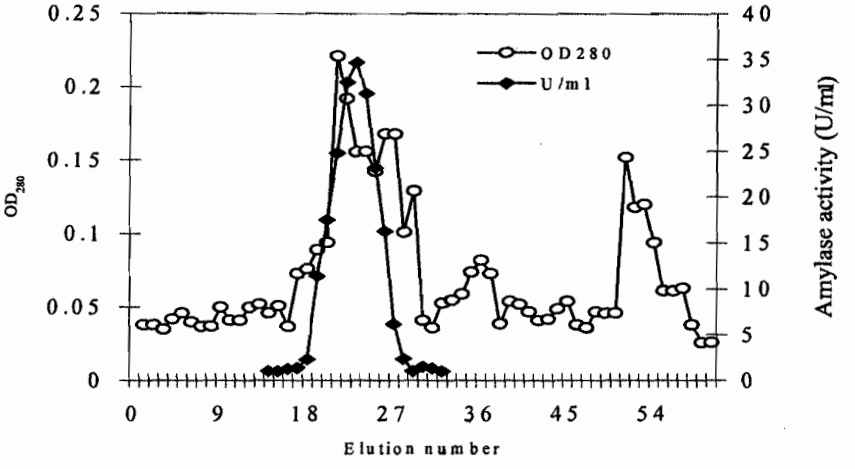

Fig. 1. Alpha amylase purification profile using gel filtration column

Table 1 Purification of thermostable alpha amylase

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor (fold)	Recovery (%)
Culture Supernatant	2380	370	6.43	1	100
$(NH_4)_2SO_4$ Precipitation	1554.8	80.6	19.29	3.00	65.33
Gel filtration (Sephadex G-100)	1107.2	5.12	216.25	33.62	46.52

Optimum temperature and thermostability

The optimum temperature for the activity of the α-amylase was determined to be 60-70°C (Figure 2). About 70% of the enzyme activity was displayed at 40°C, which implies that the enzyme can potentially be used at lower temperatures. Temperature optima for the catalytic activity of *B. stearothermophilus* α-amylases generally are 50-70°C (Wind *et al.*, 1994).

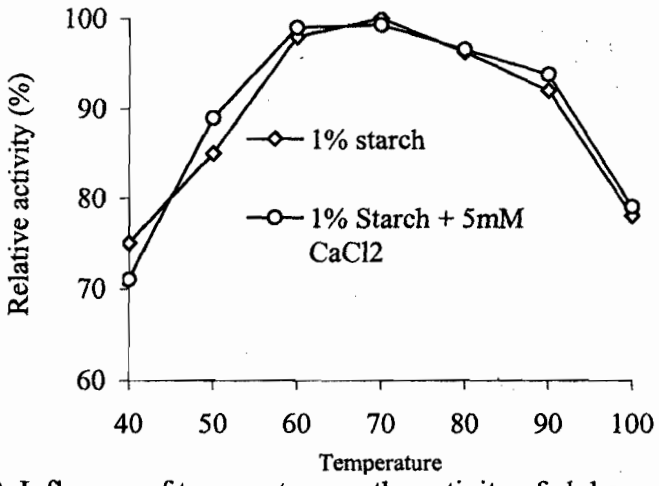

Fig. 2. Influence of temperature on the activity of alpha amylase

Thermostability experiments revealed that 50% of the original activity was maintained after 45 minutes of incubation at 80°C (Figure 3). The α-amylase from *B. stearothermophilus* GRE 1 was found to be more stable than α-amylases produced from *B. stearothermophilus* (Jeayoung *et al.*, 1989), *B. subtilis* (Canganella *et al.*, 1994), *Lactobacillus manihotivorans* (Aguilar *et al.*, 2000) and *Thermomyces langinosus* (Bo and Olsen, 1991) but less stable than *B. licheniformis* (Viara *et al.*, 1993), *B. stearothermophilus* (Wind *et al.*, 1994) and *Pyrococcus woesei* (Koch *et al.*, 1991).

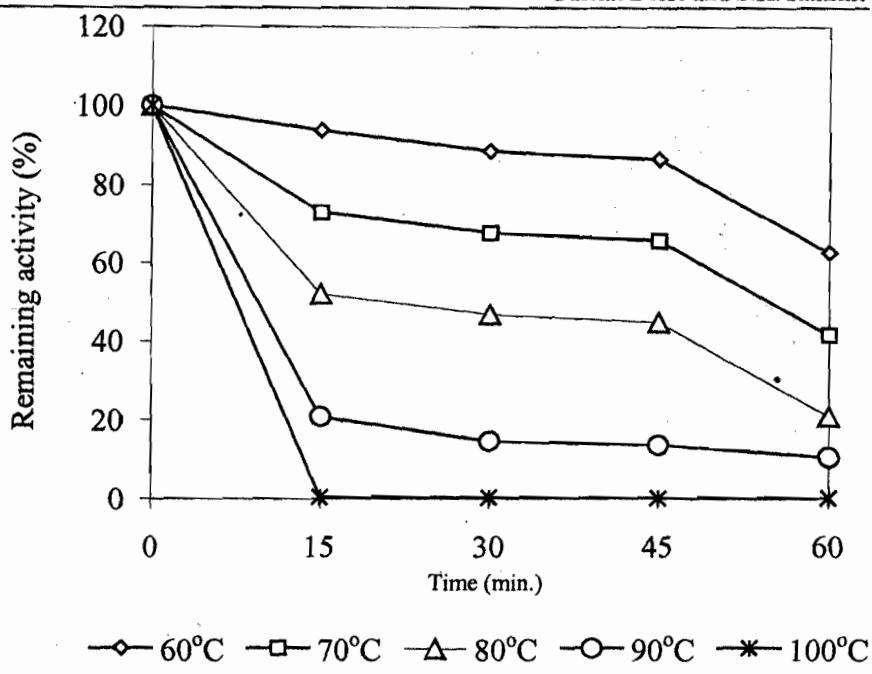

Fig. 3. Thermostability of alpha amylase

Effect of pH on activity and stability

The optimum pH of the enzyme was in the range of 5.5-6.0 in which more than 90% of its peak activity was displayed (Figure 4). Enzyme activity at the pH values of 4 and 10 was 25% and 39%, respectively. Although the optimum pH of amylases produced from various *B. stearothermophilus* was reported to vary, it generally ranges between 4.5-8.0 (Vihinen and Mantsala, 1990). Commercially α-amylase at a pH range of 6.0-7.0 is preferred. Figure 5 shows that the enzyme is fairly stable at pH values of 5.0-9.0, with 27% and 25% activity remaining at pH 4.0 and 10.0, respectively. Considering the requirement of starch liquefaction process of an enzyme with a low pH optimum, the present enzyme might offer an advantage since it is worked between pH 4.0-10.0. From an economical point of view, this allows the minimizing of acids and bases used for pH adjustment and reduction of investment on ion-exchange media and chemicals to purify the syrup produced.

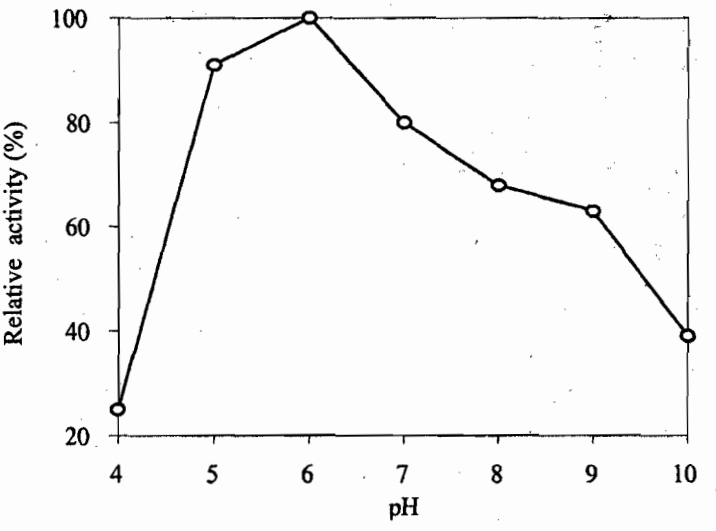

Fig. 4. Optimum pH for the amylase activity at 60°C

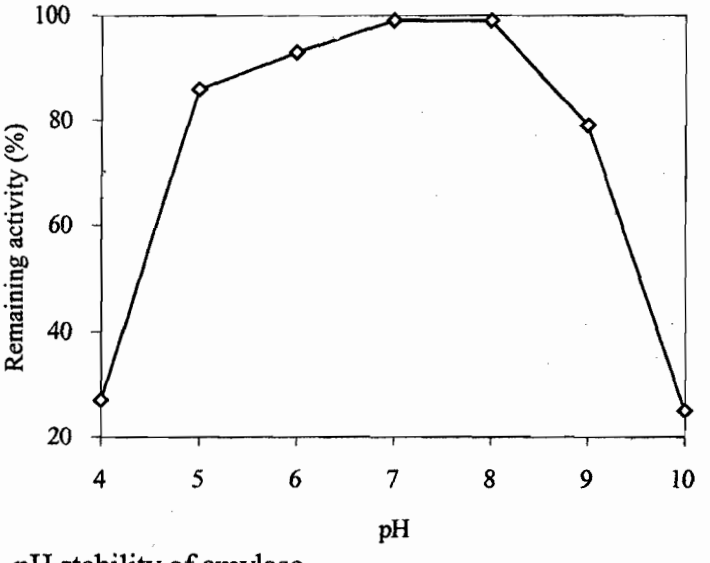

Fig. 5. pH stability of amylase

Effect of metal ions

Table 2 shows the effects of some metals on the amylase activity. The ions Cu^{2+}, Zn^{2+} and higher concentrations of Fe^{2+} strongly inhibited enzyme activity and induction by Ca^{2+} was not significant. A relative activity of 94% and 86% was displayed with low concentrations of Co^{2+} and Mg^{2+},

respectively.

While calcium in amylase is known to enhance amylolytic activity, little is known about the function of either zinc or copper, albeit both are important in biological systems (Agarwal and Henkin, 1987). Increasing the concentration of copper sulfate in the reaction mixture decreased the rate of activity. Cu^{2+} could displace Ca^{2+} in the active site, but could not be able to facilitate catalysis, thus leading to inhibition. Removal of the Cu^{2+} and its replacement by Ca^{2+} could restore activity.

Table 2 Effect of metal ions on amylase activity

Cation	Concentration (mM)	Relative Activity (%)
None		100.00
Ca^{2+}	1	97.58
	5	103.52
	10	96.11
Co^{2+}	1	94.24
	5	88.50
	10	87.97
Mg^{2+}	1	86.41
	5	85.99
	10	83.49
Fe^{2+}	1	88.29
	5	55.62
	10	44.77
Cu^{2+}	1	45.19
	5	24.52
	10	13.78
Zn^{2+}	1	43.83
	5	12.11
	10	9.60

Raw starch degradation

The enzyme hydrolyzed wheat, corn and tapioca starch granules efficiently, but had low activity on potato starch granules at 40 and 50°C. An increase in temperature to 60°C improved hydrolysis of potato starch, and promoted its degradation up to 50% after 36 h. Nearly 50% degradation rate of all the starches was obtained after 12 h of incubation at 70°C (Figure 6). Micrographs showing morphological changes of wheat and tapioca raw starches before and after digestion are presented in Figure 7.

In an effort to produce raw starch digesting thermostable alpha amylase, a *Bacillus* sp. was isolated from a hot spring (Gashaw Mamo and Amare

Gessese, 1999). The enzymes, extracted by shake flask, were further purified to yield two types of amylases (Amy I and Amy II), which absorbed and hydrolyzed potato and corn starch. A thermostable α-amylase capable of digesting raw starch was also isolated from *B. stearothermophilus* (Kim *et al.*,1989). This enzyme, which was active at 70°C, could strongly degrade starch granules up to 93% and potato starch granules up to 80%.

Strains of *B. stearothermophilus* hydrolyzed 80% of barley starch. Corn and rice starches were also hydrolyzed efficiently reaching 70 and 63% conversion, respectively. Potato starch was however hydrolyzed slowly and degradation of granules was most effective at 60°C (Campus *et al.*, 1992).

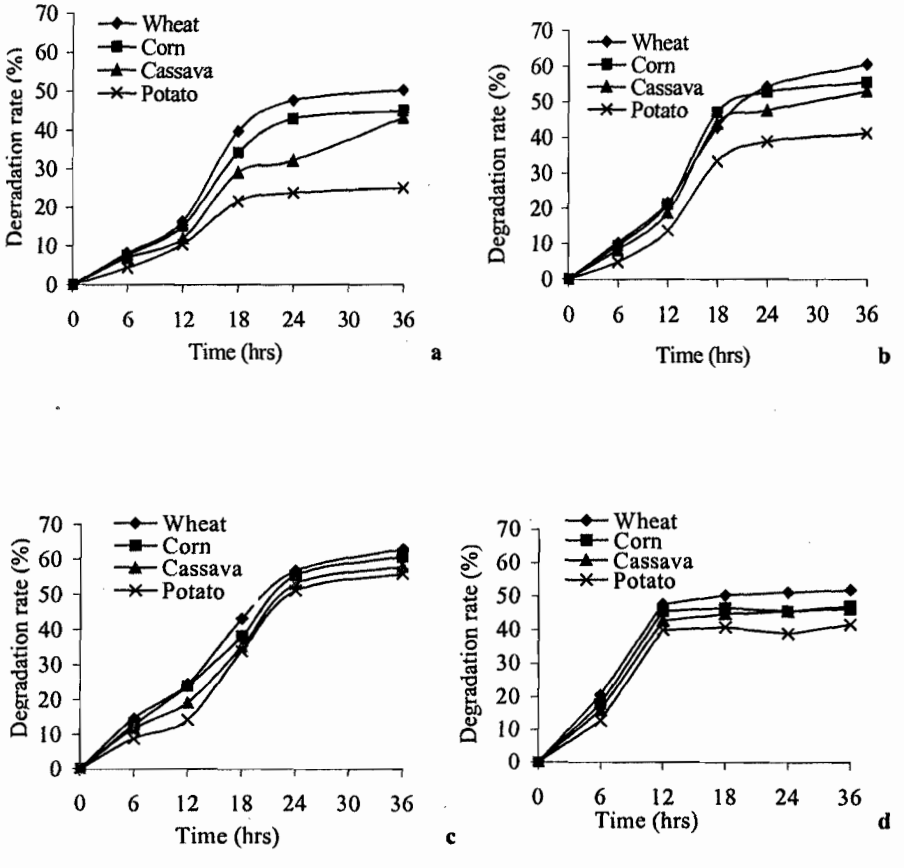

Fig. 6. Degradation rates of raw starch granules by amylase action (a) 40°C (b) 50°C (c) 60°C (d) 70°C

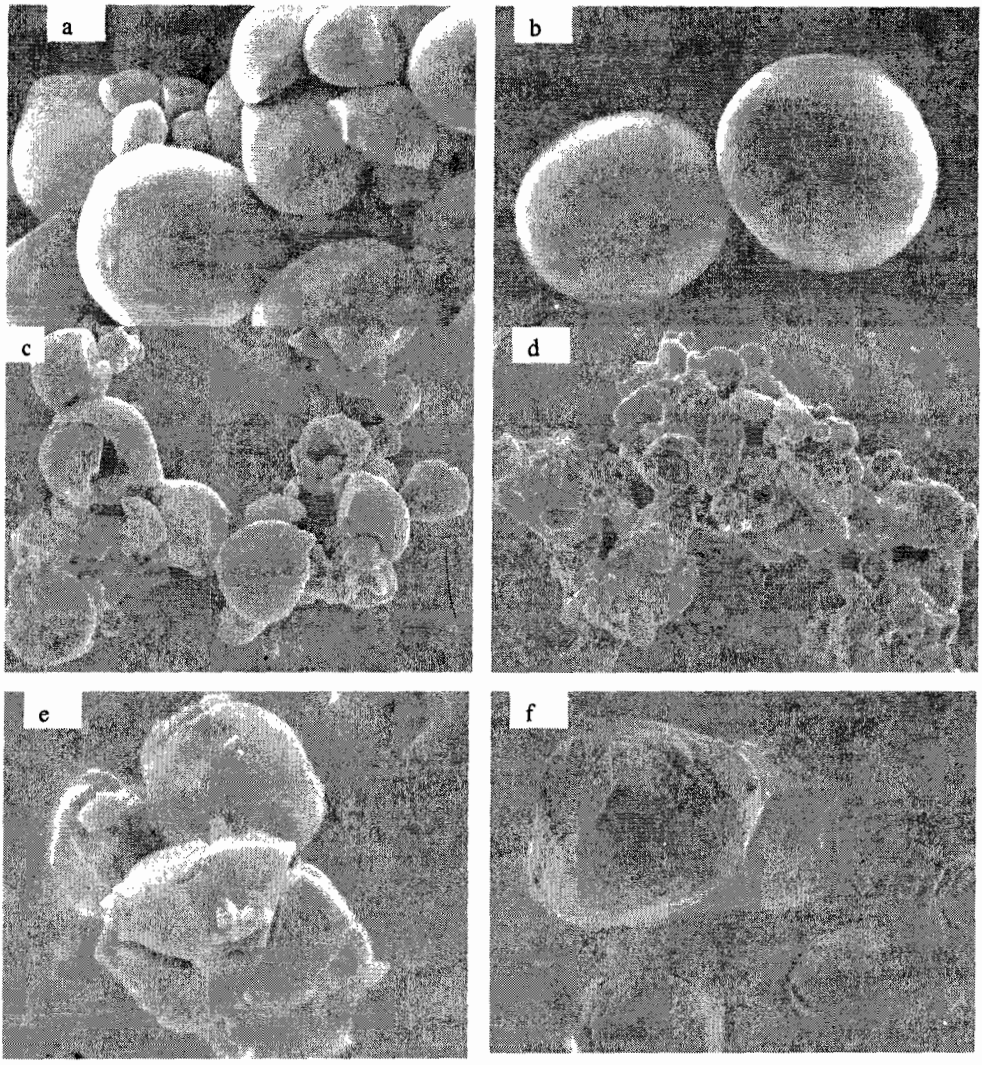

Fig. 7. Scanning electron microscopy of (a) tapioca, (b) wheat raw starches before digestion, (c) tapioca and (d) wheat raw starches digested at 40°C for 6 h, (e) tapioca and (f) wheat raw starches digested at 50°C for 12 h.

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