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UTILIZATION OF AGRO-INDUSTRIAL WASTES FOR THE PRODUCTION OF AMYLASE BY INDIGENOUSLY ISOLATED *BACILLUS CEREUS* AS2

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ABSTRACT

Amylases are starch-degrading enzymes that hydrolyze the glycosidic bonds in starch molecules to yield valuable products. Microorganisms are frequently used to produce high levels of amylase and are important commercially due to the cost effective production. Microorganisms are capable of utilizing waste materials obtained as a result of various agricultural and industrial processes to produce valuable products. Indigenously isolated *Bacillus cereus* AS2, a potential amylase producer was investigated for its capability of utilizing different agro-based natural substrates including sugarcane bagasse, wheat-bran, rice bran, maize-flour, rice-flour and potato-peels. Significant amount of amylase was achieved by using such agro industrial wastes as substrates. Maximum amount of enzyme was produced with potato peels as a substrate. Sugarcane bagasse, wheat bran and rice bran also produced significant amount of amylase as compared to commercially available starch. Amylase was also found to strongly adsorb to potato peels i.e., 72.52%, followed by starch, wheat bran and sugarcane bagasse (70.3, 67.9 and 57.1%, respectively). Scanning electron microscopy revealed the surface hydrolysis of different natural substrates by the enzyme in the form of grooves and holes. Thus these agro-wastes could be utilized as an economical and environment friendly alternatives (substrates) for amylase production.

Key-words: Amylase, agro-wastes, starch hydrolysis, natural substrates, *Bacillus*

INTRODUCTION

Amylases catalyze the hydrolysis of starch subunits to produce varied sized oligosaccharides and have a vast range of applications in fermentation, baking, brewing, beverages, food, detergent, paper and textile industry (Ahlawat *et al*., 2009). Low cost production of enzyme is required in order to fulfill the higher demands of these industries. Amylases could be obtained from microorganisms, plants and animals, but microbial source are often preferred due to vast availability and cost effectiveness in the production (Serin *et al*., 2012). Among the microbial sources, *Bacillus* species are significantly used for the commercial production of amylase.

The bulk waste from agro-industrial processes and food processing industries is not satisfactory as food or animal feed as it contains a large amount of fiber to be digested by animals. Microorganisms are capable of utilizing waste generated from different agro-industrial processes as substrates for growth, which results in the synthesis of valuable by-products (Worgan, 1976; Amutha and Priya, 2011). Enzymes with numerous applications in industries have now been produced by microorganisms from the utilization of agricultural and industrial wastes. This reduces the cost of production and increases the amount of enzyme for utilization in industry (Bharathiraja *et al*., 2011). Millet, starch, potato, wheat bran and sugarcane bagasse are commonly used natural substrates for amylase production (Sajjadd and Choudary, 2012). This paper concerns with the economical production of amylase enzyme from different agricultural wastes so that the biomass generated annually in millions of tons could be utilized.

MATERIALS AND METHODS

Bacterial strain

After the qualitative and quantitative screening of 39 different soil isolates of *Bacillus* for the extracellular amylase production, *Bacillus* AS2 was selected due to its highest enzyme activity (3179.6 IU/ml/min) (Rehman and Saeed, 2015). This strain was used in the study after optimizing different physicochemical factors for the maximum enzyme production (Rehman, 2018).

Enzyme Assay

Enzyme assay was performed as by the method of Bernfeld (1955). Reaction mixture for the determination of the enzyme was prepared by adding 100 µL of soluble starch (in 20mM sodium phosphate buffer) as a substrate to 100 µL of Cell free culture supernatant (CFCS) as a crude enzyme solution. It was kept for 15 min at 50°C. In order to stop the reaction 100 µL of 3, 5-di nitro salicylic (DNS) reagent (96mM) was added, then boiled for 15 min. It was cooled at room temperature and added by 900 µL of distilled water. By using the spectrophotometer, optical density was measured at 540 nm. Values are compared with the 'Standard Maltose Curve' for the final calculation of enzyme activity in IU/ml/min (Rehman 2018).

Natural Substrates

Sugarcane bagasse (S.B), potato-peels (P.P), wheat-bran (W.B), rice bran (R.B), rice-flour (R.F) and maize-flour (M.F) were utilized as natural starch substrates. These were dried in the air and grinded by using a kitchen grinder. The powder was passed via 100 mesh sieve and finally dried at 80ºC.

Determination of Starch content of natural substrates

Bacillus AS2 was grown in L.B broth (Luria Basal) and kept at 45°C with continuous agitation at 150 rpm for 24 h. After spinning the broth at 4000 rpm (at 4°C) for 15 min, cell free culture supernatant (CFCS) was collected. It was utilized as a crude enzyme solution. CFCS (25 µL) was added in 25 µL of Na-acetate buffer (50 mM, pH 6.0). To this mixture 50 µL of acetic acid (1M) was added. After adjusting the final volume to 2.35 mL by distilled water, 50 µL of iodine reagent was added and O.D was measured at 600nm against a blank (instead of supernatant, distilled water was used). Content of starch in each natural substrate was estimated by the help of starch standard curve (Rehman, 2018).

Physical and chemical characterization of natural substrates

Characterization of natural substrates was done according to Olayemi *et al.,* (2008) by using the parameters such as pH, moisture content and water holding capacity.

a) pH

Substrate (1g) was dissolved in de-ionized water (100 mL). By using the pH meter, pH of each substrate was determined.

b) Moisture content

Starch powder (1g) of each substrate was dried at 105°C in hot air oven for about an hour. Moisture content was calculated by determining the total weight loss after drying by using the following formula:

Moisture content = Final weight after drying (wf) / Initial weight before drying (wi) × 100

c) Water holding capacity

Substrate (1g) in powder form was dissolved in 10 mL distilled water and kept at shaking for 2 h. After standing for about 30 min, contents were centrifuged for 15 min at ×4000 g. Sediment was utilized for the determination of water holding capacity by applying the following formula:

Water holding capacity =Dry weight of starch (1g) / Weight of sediment (after centrifugation)

Submerged (SmF) and solid-state (SSF) fermentation by utilizing natural substrates

For submerged fermentation, each substrate (1% w/v) was taken in a flask. After adding the overnight culture of *Bacillus* AS2 (8%), flask was kept at 40°C with shaking (at 150 rpm) for different time periods. At regular intervals samples were collected for enzyme assay.

For solid-state fermentation, each substrate (1g) was inoculated with overnight culture of *Bacillus* AS2 (8%). Moisture content of each flask was adjusted to 80 % by adding plain broth and kept at 40°C with shaking (at 150 rpm). At regular intervals samples were collected and 10 mL of Na-acetate buffer (50mM, pH 6.0) was added. It was again kept at shaking for 30 min at and mixtures were again shaken for 30 min at 40°C and filtered by Whatman filter paper no.1. After spinning the filtrate at x5000 g (at 4ºC) for 30 min, supernatant was utilized for enzyme assay.

Determination of amylase adsorption on starch substrates

All the substrates were washed twice with de-ionized water and equilibrated in Na acetate buffer (50 mM, pH 6.0). One gram of each natural substrate was mixed with 2 mL of Cell free culture supernatant (CFCS) as a crude enzyme, with gentle stirring for 15 min (at 4°C) and centrifuged for 20 min at x3000 g. Supernatant was used for determining the enzyme activity as residual enzyme (Dey *et al.,* 2003). Percent adsorption rate (AR%) was calculated according to the following equation:

Percent Adsorption Rate (AR %) = [(A-B)/A] ×100
Where;
A = crude enzyme
B = residual enzyme

Scanning electron microscopy (SEM)

All the substrates were washed twice with de-ionized water and equilibrated in Na acetate buffer (50 mM, pH 6.0). CFCS (4 mL) was incubated with each substrate (0.5 g) at 60°C for different time periods (i.e., 30 min, 1 h and 24 h). Sodium hydroxide (1M) was added to stop the enzyme activity and kept for 15 min. Samples were centrifuged at x3000 g for 20 min and enzyme assay was performed using the supernatant. In order to adjust the pH to 6.0, Hydrochloric acid (1N) was added to the supernatant and enzyme assay was performed after spinning it again for 20 min at x3000 g to confirm that the enzyme activity has stopped. Samples for Scanning Electron Microscopy were prepared by washing each sample three times in order to remove any enzyme residue. Samples were dehydrated by adding ethanol and freeze dried by tetra-butanol and observed (Mu *et al*., 2015).

Statistical Analysis

ANOVA and Tukey's test were applied to all the tested parameters using Minitab 17. At 95% confidence limit, all values with P values < 0.05 were found statistically significant (Rehman, 2018).

RESULTS AND DISCUSION

Physical and chemical properties of natural substrates

The physiochemical properties of natural substrates are exhibited in Table 1. High water holding capacity was exhibited by potato peels and maize flour followed by sugarcane bagasse, wheat bran and rice bran. Rice flour exhibited least water holding capacity among the tested substrates. Moisture content in potato peels and maize flour was also found to be high. As far as the pH analysis is concerned, sugarcane bagasse, potato peels and rice flour exhibited slightly acidic pH while other substrates were found to have neutral pH. These substrates also exhibited high starch content and hence could be used as a good substrate for amylase production. Also the starch content in case of potato peels, maize flour and rice flour was found considerable as compared with the commercially available soluble starch and hence could be used as a cheap source for amylase production. Among the tested substrates, the properties exhibited by potato peels prove it to be the most suitable natural substrate. Potato peels as the best substrate for optimum amylase production was earlier reported by Shukla and Kar (2006). Al-Weshahy and Rao (2012) and Parawira *et al.,* (2005) also reported that high mineral and starch content, water-holding capacity and dietary-fibers in potato peels stimulates microbial growth as well as enzyme production. Maximum amylase production by the use of potato peels from different *Bacillus* sp. was also reported by Mushtaq *et al*., (2016). Al-Weshahy and Rao (2012) reported that potato peels contains high starch content (i.e., 66.8%) as compared to pectin (3.4%) and cellulose (2.2%) that's why serves among the most potent substrate for amylase production. Water holding capacity of potato peels was found to be high as compared to the wheat bran, this may be due to the large grain size that allows more water uptake. Toma *et al*.,(1979), also reported the high dietary-fiber, mineral content and water holding capacity of potato peels.

Submerged (SmF) and solid-state (SSF) fermentations by utilizing natural substrates

Bacillus cereus AS2 was found to have a potential to utilize natural as well as commercial forms of starch. Maximum enzyme was produced in case of potato peels (under SmF). As compared to the commercial starch, other substrates as wheat bran, rice bran and sugarcane bagasse were found more efficient in amylase production. Amylase units were found high in case of SmF as compared to SSF (Fig. 1a and b). Under SmF, high amylase values were achieved in case of potato peels as a substrate at 96 hrs, further increase in incubation time resulted in decrease in enzyme production. Wheat bran was also found a good substrate followed by sugarcane bagasse and rice bran. Maize flour and rice flour didn't exhibit any considerable enzyme production.

Table 1. Physiochemical characterization of natural substrates.

Natural Substrates	pH	Moisture content (%)	Hydration capacity	Starch content (%)
Sugarcane bagasse	5.7	93	1.25	0.404
Wheat bran	6.1	92	1.19	0.303
Rice bran	6.2	93	1.18	0.33
Maize flour	6.5	95	1.29	0.57
Rice flour	6.0	87	1.14	0.49
Potato peels	5.7	95	1.29	0.496
Starch	5.7	93	1.25	1.0

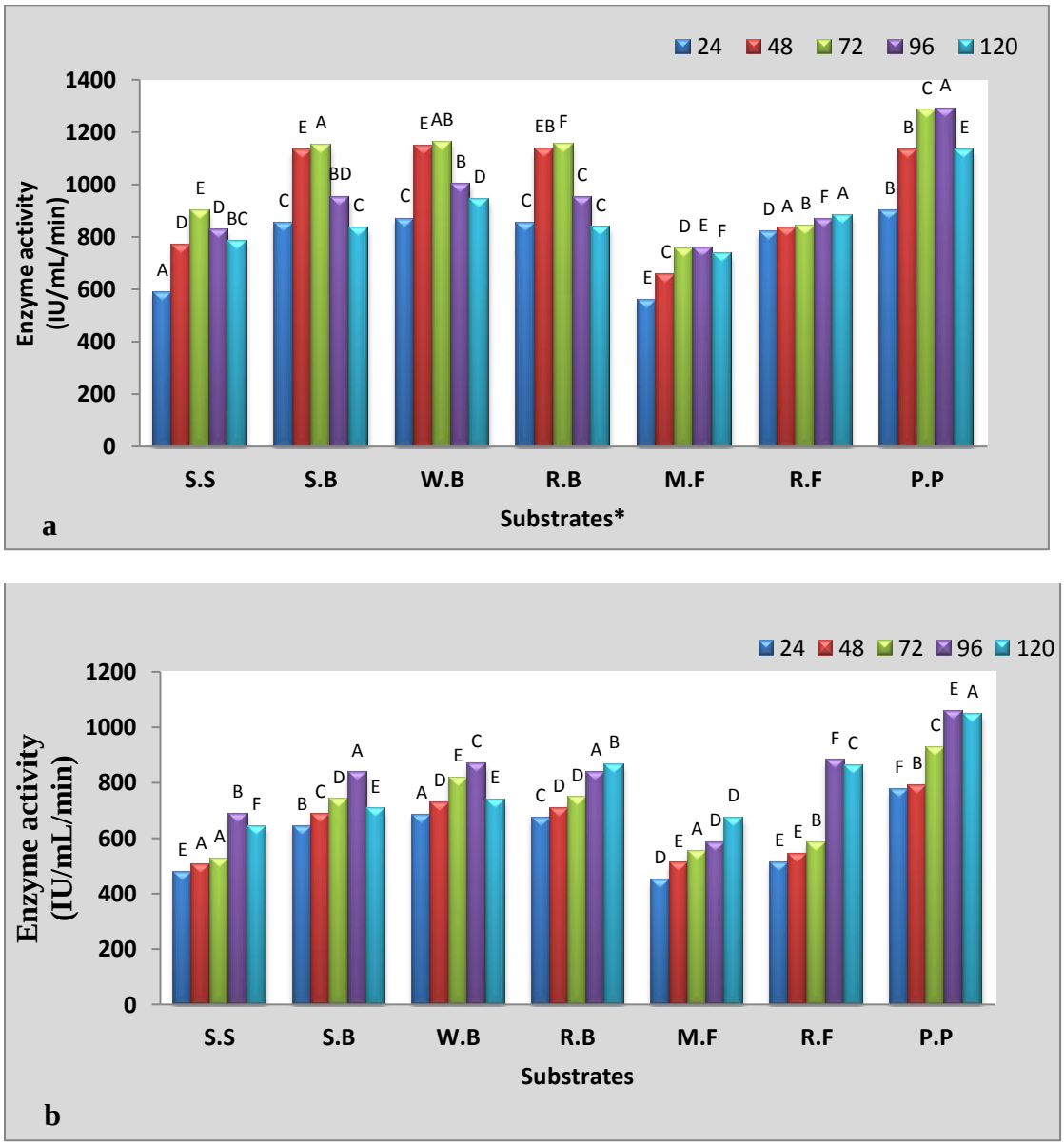

Fig.1. Effect of various substrates on amylase production by *Bacillus cereus* AS2 (a) under (a) Submerged Fermentation (SmF) and (b) Solid-State Fermentation (SSF)
(Means with different letters differ statistically as reported by Tukey's test: $p < 0.05$) (*S.S=Soluble Starch, S.B=Sugarcane Bagasse, W.B=Wheat Bran, R.B=Rice Bran, M.F=Maize Flour, R.F=Rice Flour, P.P=Potato Peels)

Under SSF, maximum amylase production was achieved by potato peels. Wheat bran and sugarcane bagasse also exhibited considerable enzyme production. The findings reported here support the cost effective production of amylase by using agro-industrial wastes as substrates. This is a very significant approach, being practiced by many workers (Bharathiraja *et al*., 2011 and Yasmin *et al*., 2017).

Adsorption of amylase on natural substrates

For determining the adsorption potential of amylase on the substrate, percent adsorption rate (AR %) was calculated (Fig. **2**). Amylase from *Bacillus cereus* AS2 was found to adsorb strongly to potato peels (AR=72.52%), followed by soluble starch (AR=70.3%) and wheat bran (AR=67.9%). Sugarcane bagasse and rice bran showed slightly lower AR values i.e., 57.1% and 50.6%, respectively. Rice flour and maize flour exhibited the percent AR less than 50 (41.4% and 40.5%, respectively). Certain openings and passages in the starch granules facilitate the entry of water, enzymes and different reagents. Amylases specifically act on starch molecules, any modification in the substrate structure would affect the substrate hydrolysis by enzymatic attack and more extensive hydrolysis (Chen *et al*., 2011).

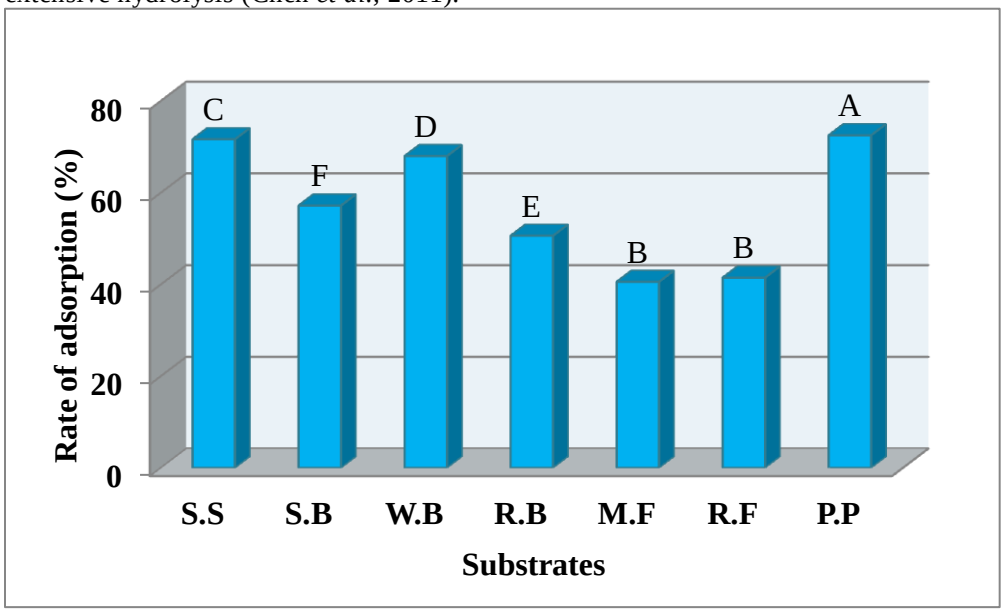

Fig.2**.** Adsorption of amylases on natural substrates produced by *Bacillus cereus* AS2 (Means with different letters differ statistically as reported by Tukey's test: $p < 0.05$) (*S.S=Soluble Starch, S.B=Sugarcane Bagasse, W.B=Wheat Bran, R.B=Rice Bran, M.F=Maize Flour, R.F=Rice Flour, P.P=Potato Peels)

Determination of substrates hydrolysis through scanning electron microscopy

Bacillus cereus AS2 was found to produce surface changes in commercial as well as natural substrates as seen in the electron micrographs (**Fig.**3). The surface changes appeared in the form of holes or crakes. The destruction on the substrate surface was enhanced by the passage of time. Pattern of hydrolysis was not found to be the same among the tested substrates might be due to the change in the structural features of every substrate. Starch granules are hydrolyzed by exo-corrosion or endo-corrosion. Exo-corrosion results in the modifications only on the exterior surface of the substrate while endo-corrosion results in the form of pores (Oates1997; O'Brien and Wang, 2008). According to SEM micrographs, wheat bran granules exhibited the hydrolysis on the external surface (by exo-corrosion), while the other tested natural substrates exhibited the surface changes in the form of pores (indicating endo-corrosion). According to Oates (1997), endo-corrosion results in the pore formation as the starch granule is corroded internally and it facilitates enzyme penetration into the substrate.

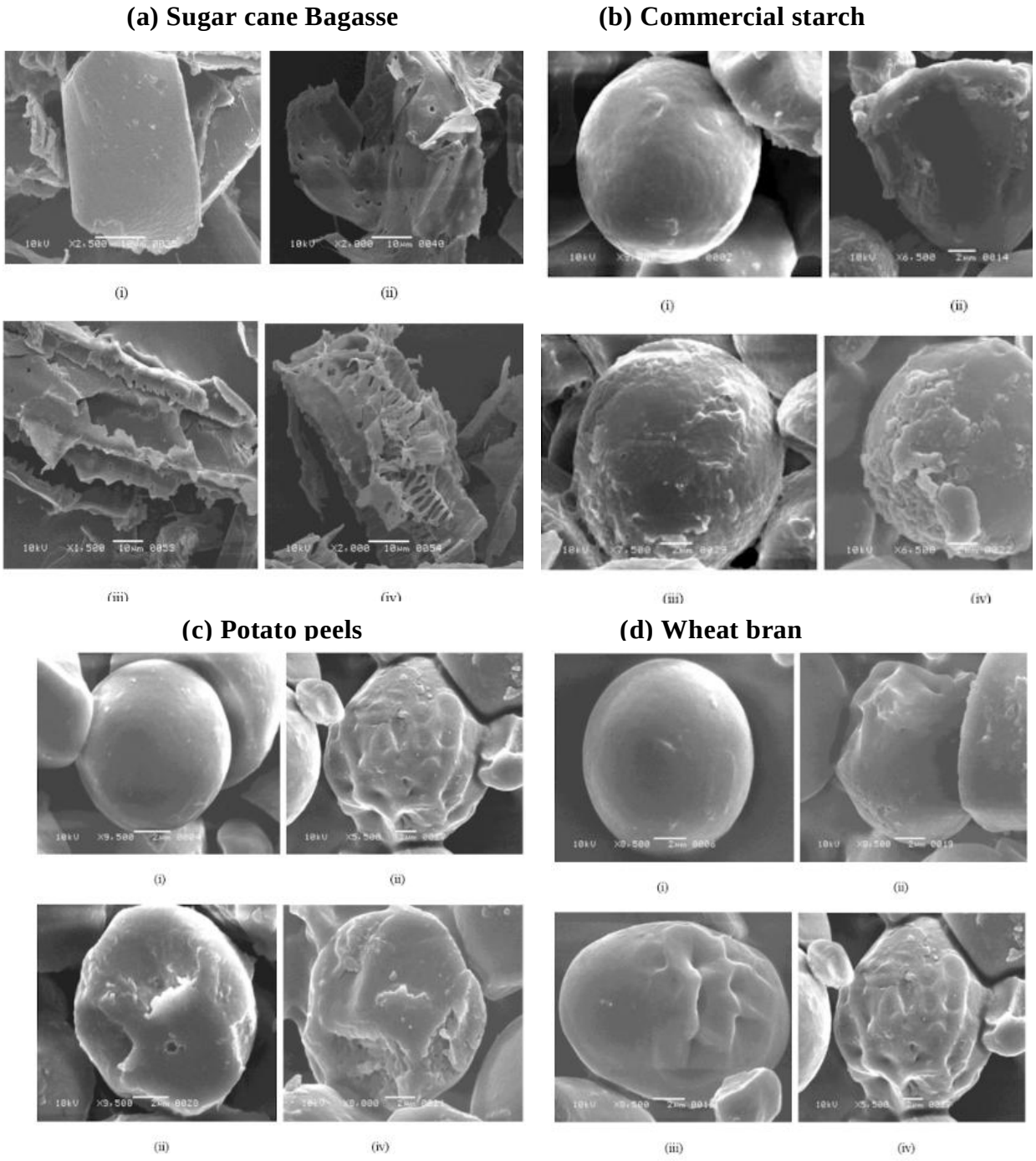

Fig. 3. Scanning electron micrographs showing amylase based starch degradation within different agro-industrial wastes at (i) 0 min (ii) 30 min (iii) 1 h (iv) 24 h.

Conclusions

Bacillus cereus AS2 was found to produce significant amount of amylase by using various agro-industrial wastes as substrates. Potato peels exhibited maximum amylase production, thus could be utilized as a cost effective and readily available source of starch. Zymo-conversion of agricultural wastes from the potential producers provides an excellent alternative of recycling of otherwise pollutant materials.

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