Chapter 4: Application of acidophilus amylase RBP 7 in saccharification of natural raw starch and different food stuff, enzyme efficacy, cytotoxicity and waste management study

4.1. Introduction

To employ any amylase as a part of the digestive syrup, it should be withstand the acidic environment of the stomach as well as have the ability to degrade starchy food stuffs even in heterogeneous condition. Alpha amylases from *Aspergillus* spp. was reported to have important role in saccharification of pure starch (Reddy and Yang, 2006; Pervez et al., 2014). Salivary amylase is generally effective at pH 6.5-7.0. Under more acidic condition, the protein structure of salivary amylase is denatured. Consequently salivary amylase does not function in stomach because stomach pH is around 2.0. Therefore, α- amylase in acidic condition which is as like as the pH of stomach has the greatest value in current days. However, to date, a few fungal amylases have been reported which possesses ability to degrade raw and natural starchy food stuffs (Forgarty and Kelly, 1979; Okolo et al., 2000; Gangdharan et al., 2006).

In this context the saccharification efficacy of raw starchy food stuff like taro, yam, malanga and sweet potato by the crude α- amylase of *Aspergillus niger* RBP7 have also been examined. Moreover the digestion of raw starchy materials and starchy materials present in nutritionally heterogeneous food by purified acidophilic amylase enzyme was carried out and also compared its activity with commercially available diastase.

Cell viability test of acidophilic amylase was done here to evaluate the occurrence of cytotoxicity by the action of the enzyme for further use of this enzyme in digestive syrup producing industries.

The application of acidophilic amylase in paper and pulp industry waste and sugarcane waste is studied here. The pH of pulp and paper industry waste are in between 4.5- 6.5. It is reached in aldehyde group, cellulose, chlorinated compounds, fatty acids, tannins, sulphur etc (Kesalkar et al., 2012). To enhance the quality, different additives and coating chemicals are used like kaolin, $CaCO_3$, talc, starch, latex etc. The pH of sugar cane waste

is between 3.5- 4.6. The sugarcane bagasse mainly composed of sucrose, lignin, aromatic molecules etc (Rezende et al., 2011).

The present chapter contains the application of acidophilus amylase RBP 7 in various sectors.

4.2. Materials and methods

4.2.1. Hydrolysis of starchy foods

Four different starchy foodstuffs taro (*Colocasia esculenta*), yam (*Dioscorea alata*), malanga (*Xanthosoma sagittifolium*) and sweet potato (*Ipomoea batatas*) were collected from market, washed thoroughly with tap water, air dried, cut into pieces (0.6 cubic mm) and crushed them by mortar pestle. Each substrate (1 g) was dissolved in 2 ml of 0.2 M of acetate buffer, followed by incubation with 3 ml of crude enzyme for varying period of time (15, 30, 45 and 60 minutes). Reaction mixture (2 µl) was spotted onto Whatman No.1 paper and subjected to descending chromatography in a solvent system [n-butanol: acetic acid: distilled water in (4:1:5) ratio]. The starch hydrolytic products were identified on the basis of R_f values after sequential treatment with 1.2% $AgNO_3$, 0.1% KOH, 5% $Na_2S_2O_3$. Here soluble starch, glucose, maltose were used as the standard.

4.2.2. Study of enzyme efficiency

Carmozyme™ (Mendine Pharmaceuticals, India), Unienzyme™ (Unichem Laboratories), Aristozyme™ (Aristo Pharmaceuticals), Vitazyme™ (East India Pharmaceuticals) were used as commercial α- amylase source for this study (Table 4.1). In first experiment to know the catalytic activity of enzyme there are different types of raw starch are used as substrate such as corn starch, wheat starch, cassava starch, potato starch, soluble starch etc. After that the raw starch were smashed with a homogenizer with the addition of acetate buffer (pH 3.0) and then the substrates (1 ml) were incubated with 1 ml of purified enzyme and different commercial enzyme preparations at room temperature. Finally after the incubation the reducing sugars of each experimental set were determined according to DNS method by Miller (1959).

In second experiment, a mixed preparation of lunch food ingredients (rice, fish and vegetable curry) was crushed into small pieces using a food processor. Then the raw material was smashed with a homogenizer with the addition of acetate buffer (pH 3.0). After that the substrate (1 ml) was incubated with 1 ml of purified enzyme and different commercial enzyme preparations at room temperature. After incubation the reducing sugars of each experimental set were determined according to DNS method by Miller (1959).

Table 4.1. Composition of different commercially available amylase enzyme

Name of the commercial amylase	Composition
Carmozyme	Alcohol 5%, fungal diastase 50 mg, hydrolysed casein 225 mg, papain 2.5 mg
Unienzyme	Activated charcoal 75 mg, fungal diastase 100 mg, papain 60 mg
Aristozyme	Pepsin 10 mg, fungal diastase 50 mg
Vitazyme	Caraway oil 500 mcg, cardamom 500 mcg, cinnamon oil 250 mcg, fungal diastase 40 mg

4.2.3. In vitro cytotoxicity assay

The cytotoxicity of purified amylase was tested. Human intestinal epithelial (InEpC) cells were seeded into 6 well culture plates with 1.7×10^6 cells/ml concentration. Purified amylase was added to each culture well at a concentration of 0µg/ml (control), 20µg/ml, 40µg/ml, 60µg/ml, 80µg/ml and 100µg/ml against control incubated at 37°C with 5% CO_2 for 48 h. Cell viability (%) was represented as visible morphological changes in Vero cells with respect to cell control.

4.2.4. Application of acidophilic amylase in waste management

The pH of pulp and paper industrial waste and sugarcane bagasse waste is between 4.5- 6.5 and 3.5- 4.6 respectively which are almost similar to the optimum pH of the isolated amylase RBP7. Therefore, the amylase RBP7 is also used in degradation of these waste materials.

In first experiment, the total solids were measured by weighing the amount of solid present in one liter of waste water (paper pulp industrial waste and sugar cane bagasse waste). At first two filter papers were dried in hot air oven at 80 ^{o}C and the weight of the filter papers were taken. Then 50 ml of waste water of each sample were filtrate through these filter papers and again the filter papers were dried. After that the weight of dried filter papers with solid substrate were measured.

In second experiment, the waste materials were homogenized with the addition of 1 ml of acetate buffer (pH 3.0). After that the homogenized raw materials (1 ml) were mixed with 1 ml of purified enzyme RBP7 and incubated at room temperature. After incubation the total carbohydrate and reducing sugars of each experimental set were determined according to Anthrone method (dissolve 200mg of anthrone reagent in 100ml of concentrated H_2SO_4) and DNSA method by Miller (1959) respectively.

4.3. Results and discussion

4.3.1. Hydrolysis of raw starchy food stuff

The digestive capability of the crude amylase of RPB7 on different raw starches was studied (taro, yam, malanga and sweet potato) (Fig.4.1 a). The conversion efficiency of each starch by α-amylase to different mono and oligo- saccharides were calculated by comparing with the glucose as standard. Results indicated that at 1 h incubation of the crude amylase was able to hydrolyze some of the raw starches tested. Yam and taro derived soluble starch used as the standard were rapidly hydrolyzed to give 100 µg/ml and 93 µg/ml reducing sugars (glucose standard) respectively. These were followed by malanga starch (49 µg/ml) and sweet potato starch (48.5 µg/ml) after 1 h of hydrolysis. Collectively the results attested that the crude amylase of *Aspergillus niger* RBP7 has immense capability to digest the raw starches (Figure 4.1 a). Calculation of the relative

conversion efficiency at 1 h showed that 10% and 9.3 % obtained for yam starch and taro starch are significantly higher than 4.9 % and 4.85 % obtained for the malanga starch and sweet potato starch, respectively. The paper chromatography analysis of the starch digest showed maltose as the predominant product of hydrolysis with small amount of glucose for all the starchy food stuff tested (Fig. 4.1b).

The appearance of maltose and low level of glucose on the paper chromatography of starch digestion by amylase of *Aspergillus niger* RBP7 indicated that the crude enzyme consists principally of α-amylase. The crude amylase of *Aspergillus niger* RBP7 selected for this study was capable of hydrolyzing raw tuber starches into maltose and glucose which can then be used directly in the production of ethanol. Maity et al. (2011) and Bozic et al. (2011) reported that α- amylases can degrade different raw starchy materials and wheat bran also hydrolyzed by α- amylase (Soni et al., 2003). Thus there is a possibility to hydrolyze raw starchy foods by using this newly isolated amylase in various application sectors.

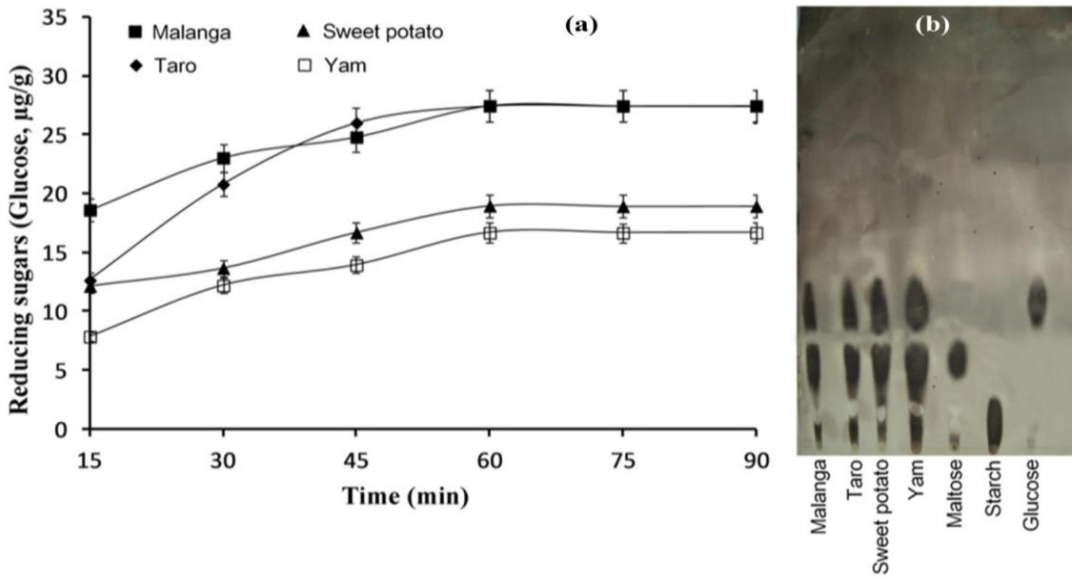

Fig 4.1. Hydrolytic capability of different raw food stuffs by acidophilus α-amylase. a) Time course study of the enzymatic hydrolysis of raw food stuffs and production of reducing sugars. b) Paper chromatography analysis of different reducing sugars produced by α-amylase after hydrolysis of raw foods stuffs

4.3.2. Comparative study on enzymatic catalytic efficiency and bioconversion of raw starch and heterogeneous nutritionally important compound

The catalytic effect of different commercially available enzymes are compared with amylase of RBP7 on different raw starch corn starch, wheat starch, cassava starch, potato starch, soluble starch etc (Table 4.2). From the experiment it was observed that Vitazyme has highest relative activity (28.23%) when it works on wheat starch where as Unienzyme has lowest relative activity (0.12%) when it works on cassava starch with respect to amylase from RBP7. In case of production of reducing sugar, Unienzyme produced 87.5 U/ml and Vitazyme produced 49.4 U/ml reducing sugar by hydrolyzing soluble starch and potato starch respectively. Degradation of starch in pure form at acidic pH is not the attestation of its application as digestive enzyme. To validate the same, lunch food ingredients was digested with the purified amylase of *Aspergillus niger* RBP7 and the performance was compared with some commercially available digestive tonic. The result revealed that liberation of reducing sugar from the meal was significantly higher after digestion with RBP7 enzyme and among the tested enzyme only Carmozyme imparted similar digestive capacity (Table 4.3). The result revealed that the amylase of *Aspergillus niger* RBP7 have immense potential to degrade starchy materials present in lunch food ingredients in heterogeneous condition.

Table 4.2. Comparison among α-amylase from *Aspergillus niger* RBP7 with the commercially available Diastase based on their catalytic activity

Enzyme	Substrate	Enzyme activity (U/ml)	Specific activity (U/mg)	Reducing sugar (µg/ml)	Relative activity (%)
α-amylase from *Aspergillus niger* RBP7 (Control)	Cassava starch	160.7 ± 0.95	5.7	80.35	100
	Corn starch	162.0 ± 0.97	5.71	81	100
	Potato starch	165.8 ± 0.82	5.9	82.9	100
	Wheat starch	170.0 ± 0.88	6.0	85	100

	Soluble starch	175.0 ± 0.92	6.2	87.5	100
Vitazyme™	Cassava starch	125.8 ± 0.95	32.3	51.6	21.7
	Corn starch	123.5 ± 0.85	31.7	50.6	23.8
	Potato starch	120.6 ± 0.95	31.0	49.4	27.3
	Wheat starch	122.0 ± 0.81	31.3	50.02	28.23
	Soluble starch	126.7 ± 0.77	32.5	52	27.6
Aristrozyme™	Cassava starch	124.3 ± 0.58	12.43	62.15	22.7
	Corn starch	126.0 ± 0.66	12.6	63	22.2
	Potato starch	130.0 ± 0.33	13.0	63.7	21.26
	Wheat starch	133.7 ± 0.49	13.4	66.9	21.4
	Soluble starch	140.0 ± 0.63	14.0	70.0	20.0
Unienzyme™	Cassava starch	160.5 ± 0.16	17.83	80.25	0.12
	Corn starch	161.6 ± 0.32	18.00	80.8	0.24
	Potato starch	165.0 ± 0.22	18.33	82.5	0.48
	Wheat starch	168.8 ± 0.17	18.8	84.4	0.70
	Soluble starch	170.0 ± 0.28	18.9	85.0	2.80
Carmozyme ™	Cassava starch	130.5 ± 0.02	17.2	62.7	18.8
	Corn starch	128.2 ± 0.09	16.9	61.5	20.9
	Potato starch	125.5 ± 0.04	16.5	60.24	24.3
	Wheat starch	129.0 ± 0.1	16.9	61.9	24.1

	Soluble starch	132.2 ± 0.07	17.4	63.5	24.5

Table 4.3. Comparison of α-amylase from *Aspergillus niger* RBP7 with the commercially available Diastase

Enzyme	Enzyme activity (U/ml)	Specific activity (U/mg)	Reducing sugar (µg/ml)
Carmozyme™	120.3	15.73	57.88
Vitazyme™	115.79	29.57	47.5
Aristrozyme™	121.73	12.17	60.84
Unienzyme™	157.36	17.39	78.65
α-amylase from *Aspergillus niger* RBP7	155.9	5.5	77.91

4.3.3. Cell viability result of acidophilic amylase RBP7

The in-vitro cell viability was evaluated in normal Human intestinal epithelial (InEpC) cell. After the treatment with purified amylase with different concentration (0 µg/ml, 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml, 100 µg/ml) there was the same number of viable cell was present (Fig 4.2). The cytotoxicity of the purified amylase was tested but it shows no significant effect on human intestinal epithelial (InEpC) cell, indicating its non-toxicity to the cells (Fig. 4.3). The cytotoxic effect of amylase was also studied by Hadrich et al. (2015) on human intestinal epithelial cell line.

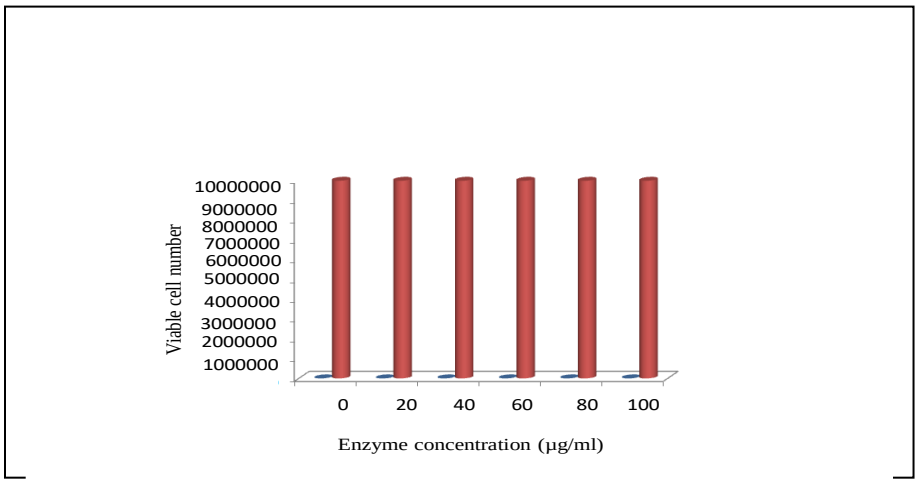

Fig. 4.2. Cell viability test at different concentration of purified amylase

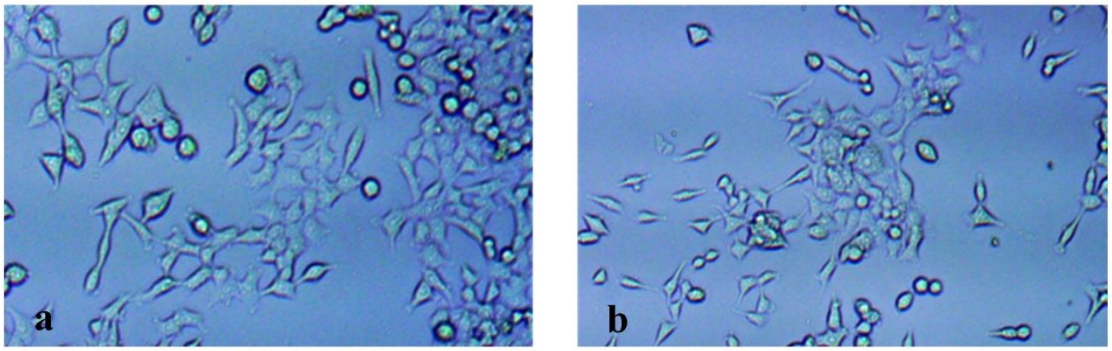

Fig. 4.3. Morphological character of Vero cell after 48h a) control and b) treated.

4.3.4. Biodegradation of different waste materials

The biotransformation of waste materials (paper pulp industrial waste and sugarcane bagasse waste) by purified amylase of *Aspergillus niger* RBP7 showed large amount of total carbohydrate, reducing sugar and total solid present in both samples. The amount of total solids in pulp and paper industrial waste is higher than sugarcane bagasse waste. The production of reducing sugar and total carbohydrate both are higher in pulp and paper industrial waste than sugarcane bagasse waste (Table 4.4). Therefore, it can be said that the amount of starchy waste is higher in pulp and paper industrial waste than sugarcane bagasse waste.

Table 4.4. Application of purified RBP7 amylase on different industrial wastes

Name of waste materials	Total solid (mg/L)	Total carbohydrate (mg/ml)	Reducing sugar (µg/ml)
Pulp and Paper industrial waste	51	77.5	73.4
Sugarcane bagasse waste	44	69.0	66.8

4.3.5. Significance of the study

The initial digestion of starch is started in the oral cavity with the help of salivary amylase. This process gives maximum effectiveness up to 30% of total starch hydrolysis (Grand et al. 2004) which indicates that the majority of starch digestion occurs in the small intestine. Any insufficiency in intestinal carbohydrate digestion is treated by the administration of exogenous digestive enzyme preparation. Most of the amylases used in digestive aids are acid-tolerant which helps them to cross the stomach environment without changing their native structure and functions. However, application of acidophilus amylases in digestive medicine can change the scenario and play some positive role by initiating starch digestion in the stomach. This process has several benefits including enhancement of stability of salivary amylase, production of maltose in the stomach that stimulates extra-gustatory sweet receptor, diminution of pancreatic load during pancreatitis, and some preventive measure against diabetic mellitus.

In our metabolic process, salivary amylase is considered as less significant in starch digestion, but the hydrolysis of starch by salivary amylase is continued in the stomach until the enzyme is inactivated at low pH (bellow 4.0) (Grand et al. 2004; Bornhorst and Singh 2012, Fried et al 1987; Freitas et al. 2017). As the postprandial gastric HCl secretion is a gradual process; thus, the rate of hydrolysis process had been maintained up to 45 minutes before strong acidification (Freitas et al. 2017).

Normally, digestive medicine has been prescribed during pancreatic insufficiency. Acidophilus amylase of Aspergillus niger RBP7 has greater potentialities to use it in the preparation of digestive medicine. As the amylase from Aspergillus niger RBP7 is active in pH 3, can start the starch hydrolysis in the stomach. The starch hydrolytic products protect the salivary amylase from pH-mediated inactivation. Thus, the activity of the enzyme has continued even at the duodenum. This action promotes the duodenal starch digestion during pancreatic disorder (Rosenblum et al. 1988). An in vitro study by Rosenblum et al. (1988) also reported that hydrolytic products of starch restore the salivary 56% of initial enzyme activity at pH 3.0. The earlier in vivo study by O. Bergeim in 1926 had established that salivary amylase has the ability to digest the mashed potatoes and bread up to 76% and 59% respectively (Freitas et al. 2018). Therefore, acidophilus

amylase along with salivary amylase makes a synergistic effect for the advancement of starch digestion in the stomach before reaching the intestine.

The initiation of polysaccharide digestion by acidophilus amylase in the stomach has another importance as the enzyme breaks the association between protein-polysaccharide (e.g. starch-gluten complex). This event liberates free protein that is cleaved by pepsin. Moreover, hydrolysis of starch by alpha-amylase produces maltose, maltotriose, and limit dextrins as the main products (Robyt 2008). Production of large amounts of maltose in the gastric environment decreases the rapid surge of blood glucose by delaying absorption at postprandial state in diabetic mellitus patients.

Butterworth et al. (2011) had reported that the expression of taste receptors gene also occurs in the stomach and other parts of the gastrointestinal tract. The sweet receptors in the stomach can be activated by maltose and release signaling peptides such as GLP-1 (glucagon-like peptide -1) and PYY (peptide YY). At the physiological levels, they regulate CNS-mediated gastric emptying, insulin secretion, and appetite (Little et al. 2009). Thus, the above-mentioned findings indicated that the application of acidophilus amylase instead of acid-tolerant amylases in digestive medicine promotes starch digestion in the stomach that has numerous significant physiological benefits.

Bornhorst GM., Singh RP. Bolus formation and disintegration during digestion of food carbohydrates, Comprehensive Reviews in Food Science and Food Safety, 2012, 11, 101-118.

Butterworth PJ, Warren FJ, EllisPR. Human α-amylase and starch digestion: An interesting marriage. Starch/Stärke. 2011, 63: 395–405.

Freitas D, Feunteun SL, Panouillé M, Souchon I, The important role of salivary α-amylase in gastric digestion of wheat bread starch. Food Funct., 2018, 9: 200-208.

Fried M., Abramson S, Meyer J.H. Passage of salivary amylase through the stomach in humans, Digestive diseases and sciences, 1987, 32: 1097-1103.

Grand RJ, MontgomeryRK, Chitkara DK, Büller HA. Carbohydrate and Lactose Malabsorption in Encyclopedia of Gastroenterology. 2004, pp. 268-274.

Little TJ, Gupta N., Maynard-Case R, Thompson DG, McLaughlin JT, Sweetness and bitterness taste of meals per se does not mediate gastric emptying in humans. Am. J. Physiol. 2009, 297: R632–R639.

Robyt JF, in: Fraser-Ried BO, Tatsuta K, Thiem J, Cote´ GL, et al. (Eds.), Glycoscience, Springer-Verlag, Berlin, Heidelberg, Germany 2008, pp. 1437–1472.

Rosenblum JL, Irwin CL, Alpers DH. Starch and glucose oligosaccharides protect salivarytype arnykzse activity at acid pH. Am. J. Physiol. 254 (Gastrointest. Liver Physiol. 1988, 17): G775-G780.

4.4. Conclusion

Paper chromatography analysis showed that hydrolysis of different food stuffs (yam, taro, malanga, sweet potato) by acidophilus amylase produced maltose and glucose. This acidophilic amylase can also hydrolyze heterogeneous food, and due to this it should be used in different digestive syrup producing industries. Moreover, this enzyme has no cytotoxic effect therefore this enzyme should be used as digestive syrup producing industry. The enzyme is also beneficial in waste management process.