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Production of Isomalto-Oligosaccharides from Available Economic Starchy Materials

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ABSTRACT:

Isomalto-oligosaccharides have a great potential to improve the physiochemical quality of many foods as anti-fading agent for food pigments, as prebiotic, food antioxidant and as a sweetener. Solid-state fermentation (SSF) has been applied in the production of many fermented foods (Couto and Sanroman, 2006). Various fungi have been used in order to produce amylolytic enzymes for starch degradation. Many fungal amylolytic enzymes are used to advantage in prebiotic oligosaccharide production. Isomaltooligosaccharides which are known as prebiotic branched-oligosaccharides have been synthesized from starchy grains (Kuriki et al, 1993; Pan and Lee, 2005). The effects of agricultural substrate and fungal strain were studied to maximize the isomalto- oligosaccharides yield. The production of Isomalto-oligosaccharides by fungal fermentation using available economic starchy materials as wheat, white corn, and starch was investigated. This study was designed to investigate the potential use of economic crops of Egypt to produce prebiotic isomalto-oligosaccharides using the process of solid state fungal fermentation. Solid state fermentation with Aspergillus Oryzae EMCC 126 was studied to increase the isomalto-oligosaccharides yield. Results show that the fermentation of wheat with A. Oryzae produced the highest concentrations of total reducing sugar (460.8 mg/g) and free amino nitrogen (20.3 mg/g) with the highest levels of amylolytic activity (87.0 U/g), α-amylase (32.5 U/g) and α-glucosidase (1.2 U/g) compared with that produced from fermentation of white corn or starch. The appropriate fermentation time for 5 days, the fermented wheat slurry was experimented on further in mashing for syrup production. The obtained wheat syrup contained the highest amounts of isomaltose, panose, and isomaltotriose, which were detected by very small amounts in the syrup of corn and starch.

Key words: *Isomalto-oligosaccharides, Functional Foods and Solid state fermentation*

INTRODUCTION

In the last three decades the functional food market has been deeply ingrained in Asia and is a fast growing segment of the food industry in the United States and Europe (Tomomatsu, 1994; Hasler, 1996, 2000; Milner, 2000; Arai, 2002; Roberfroid, 2002). Oligosaccharides are composed of between two and nine monosaccharides linked through glycosidic bonds. Non-digestible oligosaccharides are oligosaccharides that are not hydrolyzed by digestive enzymes in the gastrointestinal tract. Among these, non-digestible oligosaccharides have received the most attention (Gibson and Roberfroid, 1995; Grizard and Barthomeuf, 1999; Roberfroid and Slavin, 2000; Delzenne, 2003; Rastall and Hotchkiss, 2003; Swennen *et al.*, 2006; Mussatto and Mancilha, 2007). Isomalto-oligosaccharides (IMO), is a mixture of glucose oligomers with α-(1,6)-glucose linkages such as isomaltose, panose, isomaltotriose, isomaltopentose and highe

branched oligosaccharides.

Isomaltooligosaccharides (IMO) are produced [Hayashi *et al.,* 1994; Vetere *et al.,* 2000]. IMO has been ingested by humans for hundreds of years as they are naturally found in honey, miso, sake and soy sauce. Isomaltooligosaccharides, specifically, are glucose oligomers with α-D-(1,6)-linkages, including among others isomaltose, panose, isomaltotetraose, isomaltopentaose, nigerose, kojibiose, and higher branched oligosaccharides. IMO was tested as prebiotic and stimulated the growth of *Bifidobacterium, Lactobacillus* and are not used as substrate by *Salmonella* or *Escherichia coli* [Chung and Day, 2004]. Research on the production of oligosaccharides for foods was started between 1970–1975 in Japan and several oligosaccharides were produced on an industrial scale from the early 1980s to the late 1990s (Nakakuki, 2003). Regulation of the microbial ecology of the colon through the use of probiotics and prebiotics, has for decades gained special interest in the scientific consortium as well as among consumers (Fooks *et al.,* 1999; Kolida *et al.,* 2000; Rastall and Maitin, 2002; Lucas, 2002; Saarela et al. 2002; Manning and Gibson, 2004; Fedorak and Madsen, 2004; Rastall *et al.,* 2005; Douglas and Sanders, 2008; Vasiljevic and Shah, 2008). Prebiotics are non-digestible dietary components that pass through the digestive tract to the colon and selectively stimulate proliferation and/or activity of desired populations of bacteria indigenous to the human or animal colon in situ (Gibson and Roberfroid, 1995; Loo *et al.,* 1999; Roberfroid, 2008; Wang, 2009). However, in recent years, prebiotics tend to supersede probiotics due to various advantages such as resistance to digestive barrier, being cheaper, carrying less risks, providing new techno functionalities, and being easier to incorporate into the diet (Roberfroid, 2002; Tuohy *et al.,* 2005; Ouwehand *et al.,* 2005; Manning and Gibson, 2004; Macfarlane *et al.,* 2006). While human intestinal enzymes readily digest α-(1,4)-glycosidic bonds, α-(1,6)-linkages, particularly those linking longer polymers, are not easily hydrolyzed as they pass through the human gastrointestinal tract.

Solid-state fermentation (SSF) has been applied in the production of many fermented foods (Alexander, 1998). Various fungi have been used in order to produce amylolytic enzymes for starch degradation. Many fungal amylolytic enzymes are used to advantage in prebiotic oligosaccharide production. Isomaltooligosaccharides which are known as prebiotic branched-oligosaccharides have been synthesized from starch (Allia, et al, 1974; Barnes et al, 1972). The specific amylolytic enzyme, α–glucosidase, has been found to possess the activity of transglucosylation. This enzyme can catalyse both the hydrolysis of α-D-gluco-oligosaccharides and transfer of the glucosyl group to 6-OH of other glucosyl residues resulting in the synthesis of isomalto-oligosaccharides (Brody 1945). Isomalto-oligosaccharides have a great potential to improve the physiochemical quality of many foods as anti-fading agent for food pigments, as food antioxidant and as a sweetener. In addition, these oligosaccharides have physiological functions such as the improvement of intestinal microflora based on the selective proliferation of bifidobacteria stimulation (Buchholz and eibel. 2003; Chen et al, 2001). They are also associated with a lower risk of infections and diarrhea, and an improvement of the immune system response (Chung and Day 2004). This study was designed to investigate the potential use of economic crops of Egypt to produce prebiotic isomalto-oligosaccharides using the process of solid state fermentation.

MATERIALS AND METHODS

1. Preparation of inoculum of **fungal spores**

The suspensions of *Aspergillus oryzae spores* (EMCC 126) were prepared from a fully sporulated (7 days old) PDA slant culture using 10 ml of 0.85% NaCl solution. This spore suspension was appropriately diluted to required density. Spore concentration in the inoculum was estimated by a haemacytometer.

2. Preparation of substrate

Two economic crops (Corn, Wheat) and commercial starch were used in the experiment. The 400 g (on a dry basis) of each substrate was weighed separately into a triplicate Erlenmeyer flasks and distilled water containing 10% (v/v) of supplementing salt solution (30 ppm of CaCl2) was added and adjusted to 60% moisture level (Anto et al (2006). These flasks were mixed thoroughly and autoclaved at 121°C for 15 minutes.

3. Solid-state fermentation

The sterilized solid substrate was inoculated with one ml of the prepared inoculum. The inoculated substrates were mixed thoroughly and incubated at 30°C for 7 days. Samples of substrates were taken after incubation.

4. Mashing

The dried fermented mass was mixed into water to form the slurry of 30% w/v. One liter of the slurry was added with 0.03 g of CaCl2 and adjusted to pH 6 by using 0.1 M lactic acid. Mashing was carried out by following the method of Okafor and Iwouno (1990). The slurry was initially mashed at 50°C and allowed to stand for 30 min. The supernatant was collected and the remained flour was heated until it gelatinized at 88°C. The supernatant was returned to the cooled and gelatinized slurry, giving an overall temperature of 62°C. The mash was kept at this temperature for 60 min. The pH of the mash was tested and adjusted to 5.6 by adding a few drops of lactic acid. One-half of the mash was taken, boiled and returned to the main mash and the temperature increased to between 69 and 71°C. The mixture was kept at this temperature for 60 min. The mash was cooled and filtered using funnel and folded Whatman No. 1 filter paper. The filtered solution was finally boiled for 60 min to yield the malt syrup.

5. Measure of total reducing sugar (TRS) and free amino nitrogen (FAN)

The samples of fermented mass were diluted with distilled water and analysed for TRS and FAN according to the methods of Miller (1959) and Lie (1973) respectively.

6. Enzyme activity

Crude enzyme from the fermented mass was extracted by simple extraction. A fermented mass of 10g was mixed thoroughly with distilled water to a total extract volume of 100 ml. Contents were mixed by shaking for one hour at 30°C on a 150 rpm shaker. At the end of the extraction, the suspension was centrifuged at 7,000 rpm for 10 min. The extracted solution was measured for amylolytic activity, α-amylase activity and α-glucosidase activity.

7. Determination of amylolytic enzyme and α-amylase

The amylolytic activity was assayed according to Terashima method et al, (1994) after crude extraction of malted crops. 0.5 ml of the supernatant was added to 0.5 ml of a 1% soluble starch solution in 0.05 M acetate buffer. The sample was incubated at 60°C for 5 min and the increase of reducing sugars was measured ((Miller, 1959). One unit of the enzyme activity (U) is defined as the amount of enzyme required to liberate 1 μmol of maltose per min. The α-amylase activity was measured following the increase of reducing sugars with time. 0.5 ml of the supernatant solution was added to 0.5 ml of a 1% soluble starch solution in 0.05 M acetate buffer. The mixture was incubated at 70°C for 15 min (Sun and Henson, 1991). One unit of α-Amylase activity (U) is then defined as the amount of enzymes required to liberate 1 μmol of maltose per min.

8. Determination of α-glucosidase

The α-glucosidase activity was determined using a modified method of McCue and Shetty (2003). A standard reaction solution is prepared by mixing 0.1 ml of 9 mM p-nitrophenol α-D-glucopyranoside and 0.8 ml of 200 mM sodium of acetate buffer at pH 4.6 in a glass tube. The tubes were pre-incubated at 50°C for 5 min before addition of 0.1 ml of the enzyme extract. The tubes were incubated for 30 min. The enzymatic hydrolysis was stopped by addition of 1 ml of 100 mM sodium carbonate, and the samples were clarified by centrifugation at 13,500 rpm at room temperature for 5 min. The released p-nitrophenol in each sample was determined by measuring the absorbance at 400 nm compared with the blank. A standard curve was established using pure p-nitrophenol dissolved in sodium acetate buffer. One unit of α-glucosidase activity is defined as the amount of enzyme that releases 1 μmol of p-nitrophenol per min at pH 4.6 and 50°C.

9. Determination of sugars by high performance liquid chromatography (HPLC)

The samples of sugars and oligosaccharides were diluted and analyzed by HPLC using a Inertsil NH2 column (5 μm, 250×4.6 mm, Shimadzu, Japan) maintained at 40°C. The injection volume was 20 μl, and the flow rate 1.2 ml/min. The elution of sugars was carried out with 75% acetronitrile with detection with a differential refractometer (RID-10A, Shimadzu, Japan)

Results and Discussion:

Table (1) Different measured parameters in the produced syrup: [a]

Components	Wheat	Corn	Pure starch
Starch mg/g	619.3 ± 3.90 [c]	706.3 ± 5.5 [b]	906 ± 6.5 [a]
Total Reducing Sugar (TRS) mg/g	460.8 ± 1.10 [a]	220.5 ± 2.10 [b]	112.8 ± 1.90 [c]
Free Amino Nitrogen (FAN) mg/g	20.3 ± 0.85[a]	11.5 ± 0.65[b]	4.0 ± 0.40[c]
Amyl lytic activity U/g	87.0 ± 3.0[a]	48.75 ± 1.50[b]	26.0 ± 0.90[c]
α- Amylase U/g	32.5 ± 1.04[a]	6.0 ± 0.40[b]	2.95 ± 0.16[c]
α- Glucosidase U/g	1.2 ± 0.04[a]	0.75 ± 0.06[b]	0.40 ± 0.04[c]
Glucose g/l	4.2 ± 0.06[a]	1.72 ± 0.07[b]	1.30 ± 0.06[c]
Maltose g/l	0.20 ± 0.04[a]	0.08 ± 0.01[b]	0.04 ± 0.004[c]
Isomaltose g/l	0.02 ± 0.001[a]	0.01 ± 0.001[b]	0.001 ± 0.001 [c]
Panose g/l	0.005 ± 0.001[a]	0.003 ± 0.001[a]	0.002 ± 0.001[a]
Isomaltotriose g/l	0.006 ± 0.001[a]	0.004 ± 0.001[a]	0.003 ± 0.001[a]
Decreasing of pH*	Rapid	Rapid	Slow

*Decreasing of pH from 5 -5.6 to 4-4.5

1. **Solid-state fermentation with *Aspergillus oryzae***

IMO does not constitute a dietary fibre and instead should be explored as a slow-digesting carbohydrate (Ryan et al, 2018). Initial moisture content of each substrate was 60%. All SSF were inoculated with 1% of the prepared inoculum having 108 spores/ml and maintained at 30°C. Results in table (1) show that the highest concentrations of TRS (460 mg/g) and FAN (21.1 mg/g) were obtained from SSF of wheat. The concentration of TRS in SSF using wheat is higher than that in SSF using corn or commercial starch as a substrate together with the amylolytic activity and the activities of α-amylase and α-glucosidase. The initial pH decreased from 5.6 to 4.0 in both SSF through a 7-day period. The grains are hydrolysed by fungal amylolytic enzymes and protease resulting in increases in TRS and FAN content. The decrease of FAN and TRS after the 5-day fermentation could be due to the consumption by the synthesis of fungal biomass. The other reason of TRS decrease may be apparently due to the decrease of amylolytic enzyme (table 1). Yanfang et al.(2009) reported that amylase decreased after 5 days of SSF due to enzyme denaturation. This could affect amount of TRS. A decrease in pH level was also observed. This could be due to the production of fungal metabolites. During fermentation starch was degraded by amylolytic enzymes produced from fungi to release smaller sugars and oligosaccharides. Comparing between two substrates, wheat and corn, significant difference in the levels of amylolytic enzyme and α-glucosidase were observed after fermentation. Table (1) shows the highest concentrations of amylolytic activity, α-amylase and α-glucosidase were obtained in SSF of wheat with the value of 87.0 U/g, 32.5 U/g and 1.2 U/g respectively. The highest level of amylase was for wheat grains (32.5 U/g) compared to that of corn (6.0 U/g) and starch (2.5 U/g). This might be due to the amylolytic activity. Fogarty (1994) reported that the main amylolytic enzymes in *Aspergillus* spp. were α-amylase, amyloglucosidase, and α-glucosidase. a-Amylase is the key enzyme in starch degradation. a-Amylase hydrolyses α-(1,4)-glucosidic linkages in amylose and amylopectin and release malto-oligosaccharides of varying chain lengths while amyloglucosidase is an exo-acting starch-degrading enzyme that produces glucose from the non-reducing chain ends of the amylose and amylopectin. a-Glucosidase catalyses liberation of glucose from non-reducing ends of oligosaccharides and polysaccharides. This enzyme is able to transfer sugar moieties or groups of sugar residues from one compound to another with the formation of a similar or a distinct type of linkage. Thus, an α-(1,4) link in a chain might be broken and the separated end could be joined to the same or different chain via either and α-(1,4) or α-(1,6) link to produce molecules of maltose, isomaltose, panose, isomaltose or long chain of oligosaccharides.

3.2 Production of the rice syrup

After 5 days of fermentation, starch molecules of wheat and corn were hydrolysed by fungal amylolytic enzyme to produce small molecules of sugars and oligosaccharides. Table (1) shows that wheat slurry contain high concentration of glucose (4.2 g/l) and small amounts of maltose, isomaltose, maltotriose and panose whereas corn slurry contains small amounts of glucose, maltose, maltotriose, panose and isomaltotriose. To avoid dissolution, the fermented slurry was used directly in mashing. The

appropriate control conditions such as pH and temperature was applied in the process in order to stimulate the action of enzymes. During mashing the amylolytic and proteolytic enzymes can move freely in the liquid medium and have enough time for full starch and protein hydrolysis. The amylolytic enzymes continue to hydrolyse the remaining starch and release large amounts of fermentable sugars, in particular maltose and glucose. An increase in the levels of isomalto-oligosaccharides (isomaltose, panose and isomaltotriose) was observed after mashing. This could be due to the transglucosidase activity generated by *A. oryzae*. Comparing between two syrups, rice syrup contained the largest amounts of fermentable sugars, especially glucose and maltose. The highest levels of isomalto-oligosaccharides (isomaltose, panose and isomaltotriose) were also observed in wheat syrup.

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