Adhesion and Utilization of Native Starch Granules by *Lactobacillus amylovorus*

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ABSTRACT

This study investigated the correlation between adhesion and utilization of native starch granules by Lactobacillus amylovorus TISTR 1110 (L. amylovorus). Starch granules were used in place of a standard carbon source in the culture medium. To test whether the starch granules were still intact after media preparation, the shape, size, and birefringence of native starch granules of glutinous rice starch (GS), corn starch (CS), potato starch (PS), and mung bean starch (MS) were examined using an optical microscope under normal and polarized light. The results revealed that after treatment at a prepared temperature of 47±2°C, the morphology of starch granules at 2% (w/v) suspension did not change. The granules remained intact and maintained their birefringence. These starch granules were further evaluated for their potential to be hydrolyzed by L. amylovorus. L. amylovorus only consumed and produced a clear zone on the medium plates containing GS or CS. This hydrolysis was confirmed by observing the morphological change of the starch granules to a porous network using scanning electron microscopy (SEM). Adhesion of L. amylovorus to the native GS, CS, PS, and MS granules was also performed to verify the relation between adhesion and starch hydrolysis. High adhesion of the bacteria was found with GS and CS granules, approximately 90% of 2% (w/v) of the starch suspension in PBS pH 7.0. These results were confirmed by Gram staining.

In conclusion, L. amylovorus adhered and hydrolyzed the GS and CS granules better than the PS and MS granules. Given this, GS and/or CS offer potential as prebiotic ingredients in nutraceutical products.

Keywords: Native starch, *Lactobacillus amylovorus,* Adhesion, Prebiotic, Probiotic

INTRODUCTION

In 1999, Naidu et al. introduced the concept of probiotic as a cellular complex of lactic acid bacteria (LAB) that has the capacity to interact with the host mucosa and may beneficially modulate the immune system independently of the viability of LAB. Most probiotic bacteria used today belong to the genera *Lactobacillus* and *Bifidobacterium* (Fric, 2007). These bacteria are increasingly being included as functional ingredients, particularly in dairy products such as yogurts and fermented milks, as evidence accumulates that they have beneficial effects on human health (Macfarlane and Englyst, 1986).

Lactobacillus amylovorus is one of the amylolytic lactic acid bacteria (ALAB) that can ferment different types of amylaceous raw materials such as corn (Nakamura, 1981), potato (Chatterjee et al., 1997), or cassava (Giraud et al., 1994). *L. amylovorus*, isolated from corn-manure enrichments, produces lactic acid from starch in the form a racemic mixture of L-and D-lactic acid (Nakamura, 1981).

A prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve the hostís health (Gibson and Roberfroid, 1995). Resistant starch is that fraction of starch, which escapes digestion in the small intestine, and may be digested in the large intestine (Englyst et al., 1992). Resistant starch is classified into four types (Brown *et al*., 2004). RS1 is physically inaccessible to digestion due to entrapment in a non-digestible matrix such as is found in seeds, legumes, tubers, and unprocessed whole grains or is a digestible resistant starch due to the presence of an intact cell wall. RS2 describes native starch granules that occur in its natural granular form. This type of starch is protected from digestion by the conformation or structure of the starch granule as in raw potato and green banana. RS3 refers to non-granular starch derived materials that resist digestion. They form when starch-containing foods are cooked and cooled such as in bread or cornflakes. RS4 describes a group of starches that have been chemically modified, including starches that have been esterified or cross-linked with chemicals in such a manner as to decrease their digestion by human enzymes within the digestive tract. Granular starches synthesized by a number of food plants provide examples of such resistant starches, and they are incompletely digested due to their size and molecular conformation (Vonk et al., 2000). For this study, native starch granules from GS, CS, PS, and MS were selected as potential prebiotics.

It has been reported that some intestinal bacteria can adhere to starch *in vitro* and that adhesion is sometimes required for efficient utilization of the substrate (Reeves et al., 1997; Tancula et al., 1992). The objectives of this study were to examine the correlation between starch utilization both in solid and liquid starch culture medium and the ability of bacteria to adhere to various types of native starch granules and to investigate the morphology changes of starch granules after hydrolysis by *Lactobacillus amylovorus.*

MATERIALS AND METHODS

Materials

Native granular starch, including glutinous rice starch (GS), was supplied by the Cho-Heng Company, Thailand. Mung bean starch (MS) came from the Sitthinan Company, Thailand. Corn starch (CS) and potato starch (PS) came from Fluka, Switzerland. They were used as the carbon source, instead of glucose, for growth of *Lactobacillus amylovorus*. All other chemicals used in the experiments were purchases from Merck, Germany. The *L. amylovorus* used in this experiment was obtained from the Thailand Institute of Science and Technology Research.

Microscopic studies of native starch granules

The granular structure of GS, CS, PS, and MS were observed before and after they were added to the modified culture medium of starch agar and cooled to 47±2°C after autoclaving. They were prepared on a microscope slide and viewed under normal and polarized light.

Starch hydrolysis

The capacity of *Lactobacillus amylovorus* to hydrolyze starch granules was investigated using agar plates containing the various types of starches. The growth medium contained beef extract, 8 g/l; yeast extract, 4 g/l; peptone, 10 g/l; K_2HPO_4, 2 g/l; $MgSO_4.7H_2O$, 0.02 g/l; $MgSO_4.4H_2O$, 0.05 g/l; sodium acetate 5 g/l; sorbitan monooleate, 1 g/l; tri-ammonium citrate, 2 g/l; agar 15 g/l; and starch, 20 g/l. The native starches were added to the medium when the medium was cooled to 47±2°C after autoclaving. The single colony of *L. amylovorus* was streaked onto the starch agar and incubated for 5 days. The potential to grow on starch agar was characterized as follows: - (no growth), + (little growth), + + (moderate growth), + + + (strong growth), and + + + + (strong growth with clear zone) when compared to growth of bacteria on glucose agar. Determination of starch hydrolysis by the bacteria was based on the procedure described by Michael and Pelezar (1995). A clear zone surrounding a bacterial growth streak line indicated starch hydrolysis. Iodine solution was added to stain the starch dark blue to observe the clear zone of starch hydrolysis.

In addition, starch utilization was studied in broth medium containing the same formula as the agar plates. The native starches (2.0% w/v) were mixed to the broth medium while the temperature decreased to approximately 47±2°C after autoclaving. Broth medium plus glucose served as the control group. The single colony of bacterial cells on MRS agar was transferred into the separate broth medium bottle composed of various types of native starches and incubated at 37°C for 0, 1, 3, and 5 days. After incubation at each time interval, the samples were centrifuged at 4°C, 5,000 rpm for 10 min. Culture supernatant was taken to measure the pH change at various times and the starches remaining were lyophilized to study the morphology change of the starch granules after hydrolysis by bacteria using scanning electron microscope (SEM).

Adhesion to starch granule

For the adhesion assay, *L. amylovorus* was grown on MRS broth and incubated at 37°C for 18-20 hours. The cells were harvested by centrifugation at 4°C, 5,000 rpm for 10 min and washed three times with 0.1 M phosphate buffer saline (PBS, pH 7.0) before they were re-suspended in the same buffer at a concentration of approximately 10^7 cell. ml^{-1} and used for the adhesion assay. This study used concentrations of 0.5, 1.0, 1.5, and 2.0% (w/v) of starch suspension of GS, CS, PS, and MS. Two milliliters of the bacterial suspension was mixed by the vortex mixer with an equal volume of a suspension of starch in PBS (pH 7.0) and allowed to sediment at room temperature for one hour. After sedimentation, the supernatant was taken from below the liquid surface and the optical density at 540 nm was measured. The adhesion of bacterial cells to starch granules was determined by using the equation as described by Crittenden et al. (2001). Light microscopy was used to confirm the binding of cells of *L. amylovorus* to starch granules stained with crystal violet solution.

Statistical analysis

Results were expressed as the average of three independent experiments of mean ± standard deviation. The difference of the mean between the test groups was compared by one-way analysis of variance (ANOVA) followed by Post-hoc analysis. P values less than 0.05 were considered significant.

RESULTS

Microscopic studies of native starch granules

The purpose of this study was to investigate the morphology changes of native granular starches before and after mixing in modified MRS agar at 47±2°C after autoclaving with a microscope using both normal and polarized light. As shown in Figures 1 and 2, the granular structure of GS, CS, PS, and MS before and after treatment did not change.

Under normal light, all starch granules before and after treatment had a variety of shapes, such as round or elongated ovals, depending on the type of starch or the botanical origin.

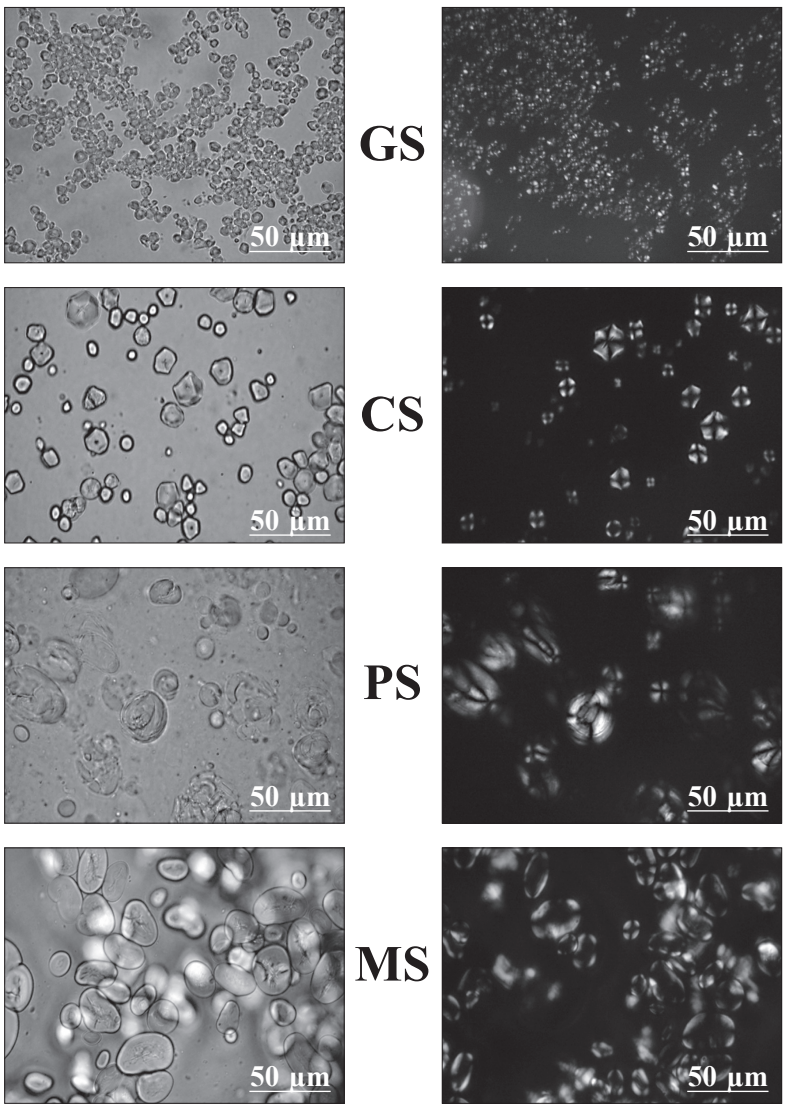

Figure 1. Light microscopy (40x) magnification of native starch granules before treatment. The left column is under normal light and the right column under polarized light.

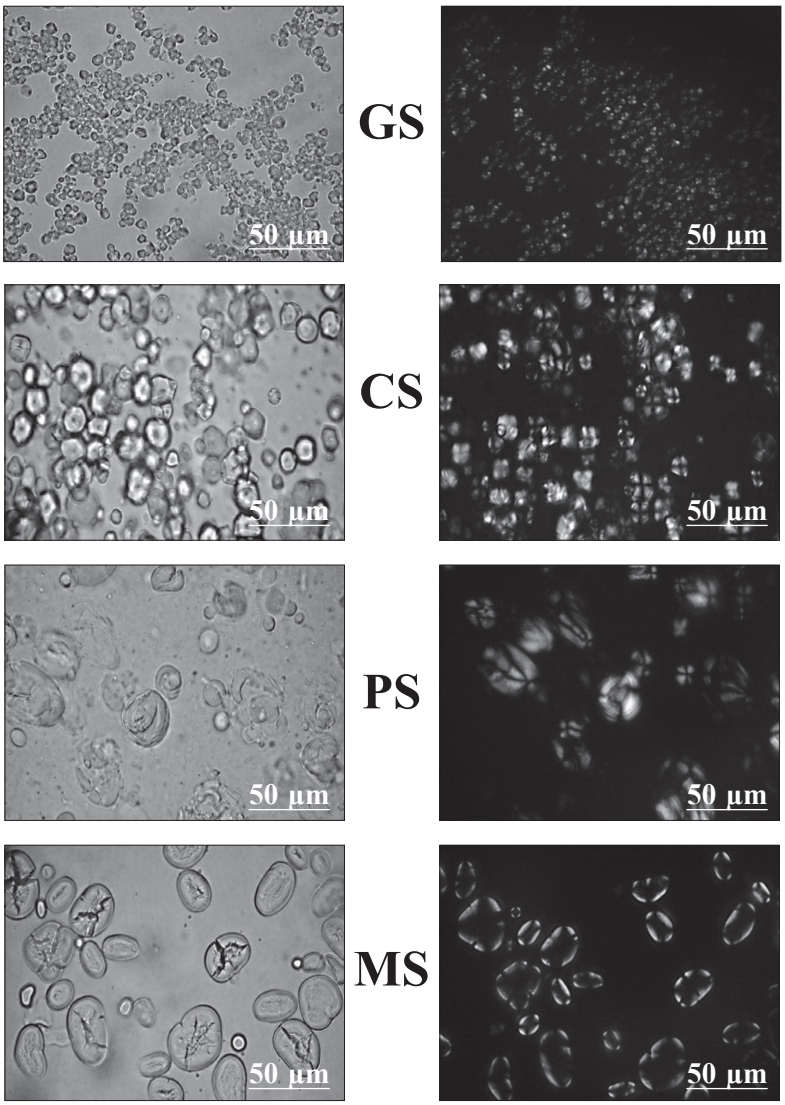

Figure 2. Light microscopy (40x) magnification of native starch granules after treatment at 47±2°C. The left column is under normal light and the right column under polarized light.

Under polarized light, eccentric birefringence and maltese cross patterns were observed both before and after treatment for the four types of starch granules used in this study due to the formation of a crystalline structure. At the 47±2°C temperature of medium preparation, the starch granules remained intact with no apparent morphological changes in granular structure.

Starch hydrolysis

The *Lactobacillus amylovorus* used in these experiments grew in the four types of starch agar plates (Table 1). However, it grew best on the GS and CS, producing a large clear zone around the colony, when compared to glucose, PS and MS.

Table 1. The capacity of *L. amylovorus* to grow and hydrolyze starch on the starch agar plates (2% w/v).

Substrate	Growth
Glutinous rice starch	+ + + +
Corn starch	+ + + +
Potato starch	+ + +
Mung bean starch	+ +

*The results were from triplicate experiments. Results were categorized as: – (no growth), + (little growth), + + (moderate growth), + + + (strong growth), and + + + + (strong growth with clear zone).

The *L. amylovorus* tested in this study appeared able to hydrolyze GS and CS. To verify this starch hydrolysis, iodine solution was added to the GS and CS agar plates. The results are shown in Figure 3. The large clear zone surrounding the bacterial growth streak line did not change. In contrast, the dark blue colour was observed on the starch agar around the clear zone that had not been hydrolyzed by bacteria.

The culture pH values ranged from 7.00–7.20 at time zero. After incubation at day 1, all of the cultures showed a decrease in pH. The fall in pH observed for all types of native starches and glucose at day 3 and day 5 were in the range of 4.1–4.70 ($p < 0.05$) compared with the day 0 experiment (Fig. 4).

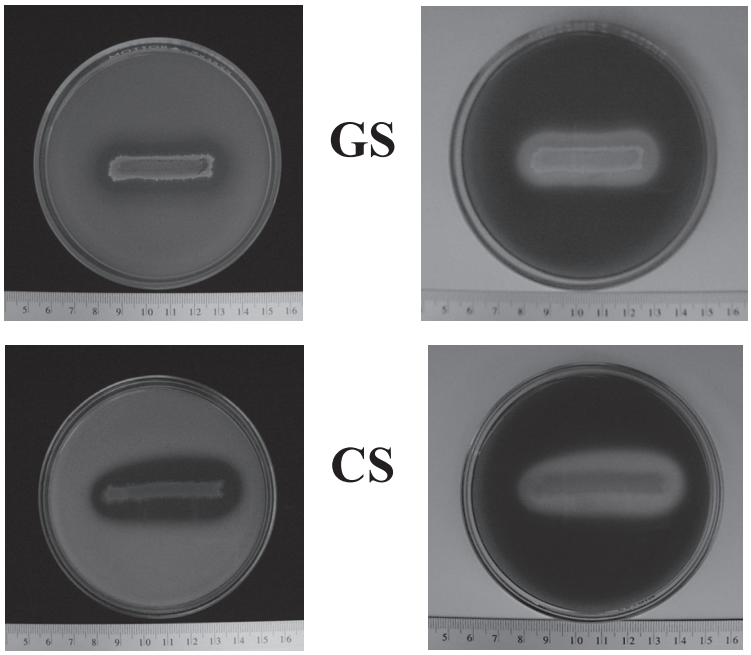

Figure 3. The ability of *L. amylovorus* to hydrolyze GS and CS by producing a clear zone around the colony on starch agar plates (2% w/v). The left column shows before and the right column after adding iodine solution.

SEM studies were used to observe the granular structure of native starch after incubation in broth medium with *L. amylovorus* as shown in Figure 5. At time 0, GS appeared to have smaller granules and to form clusters. Conversely, CS was larger and single granule. After incubation at day 5, the granular structure changed due to hydrolysis of the starch by *L. amylovorus*. The smooth surface and granular structure disappeared. Instead, irregularly shaped granules with a porous network around were seen in both GS and CS. In addition, the morphology change of PS and MS granules after incubation with *L. amylovorus* in broth medium for a period of five consecutive days looked like GS and CS (Figure not shown).

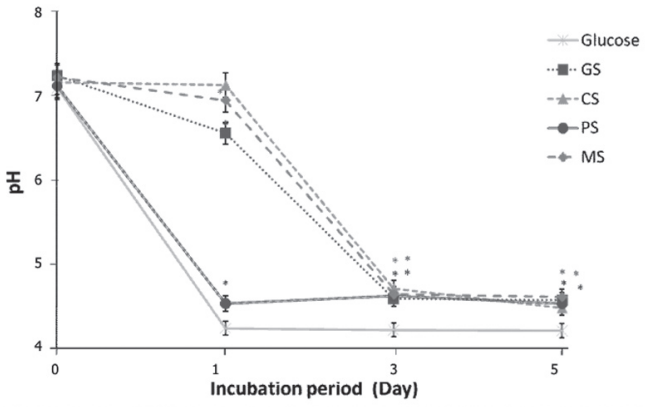

Figure 4. The pH change during incubation of GS, CS, PS, and MS in broth medium with *Lactobacillus amylovorus*.

Notes: Glucose served as the control. * Indicates significant difference ($p < 0.05$) compared to day 0 in the same group.

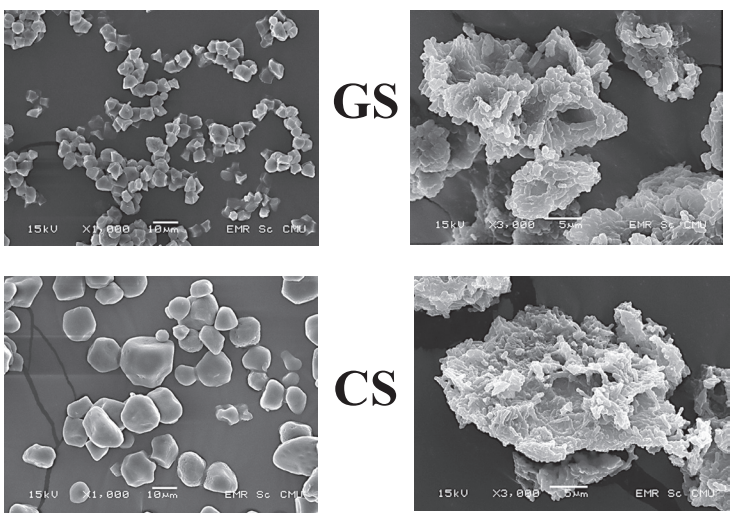

Figure 5. Scanning electron micrographs of GS and CS granules incubated in broth medium with *L. amylovorus*. The left column shows the granular structure at day 0 (x1000 magnification) and the right column the granular structure at day 5 (x3000 magnification).

The ability of Lactobacillus amylovorus to adhere to the starch granules of GS, CS, PS, and MS was also examined. The number of bacterial cells adhering to starch granules is shown in Figure 6. Moderate and high adhesion of *L. amylovorus* cells binding to starch granules appeared when the starch concentrations (w/v) increased from 0.5% to 2.0% in all cases. The maximum binding of bacteria occurred at 2.0% (w/v) with GS and CS and the adhesion percentage reached about 90%, significantly more than PS and MS (about 60-70%).

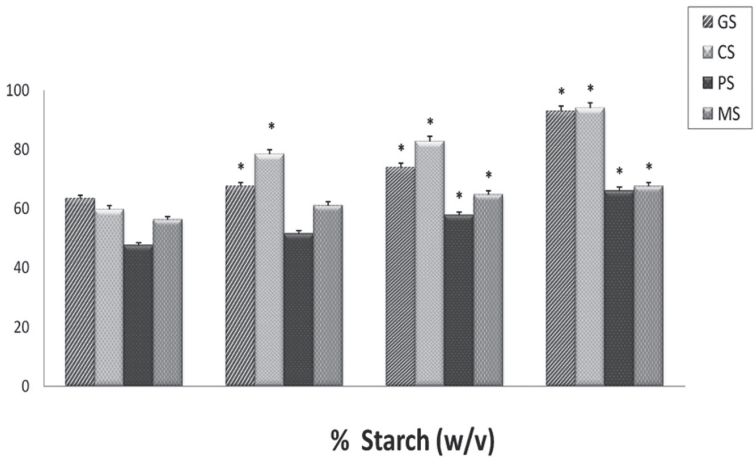

Figure 6. Adhesion of *Lactobacillus amylovorus* to suspensions of various concentrations of GS, CS, PS, and MS. * Indicates significantly different ($p < 0.05$) compared with 0.5% (w/v) in the same group.

These results were confirmed by Gram staining and then viewing under a light microscope (Fig. 7). Results indicated that the number of bacterial cells found on each starch granule varied. Some granules had bacterial cells attached to them whereas others had none. This observation may reflect the correlation between the ability to adhere to the starch granules and to hydrolyze them. It appears that the ability of *L. amylovorus* to adhere to GS and CS promoted the bacteria to hydrolyze the starch granules.

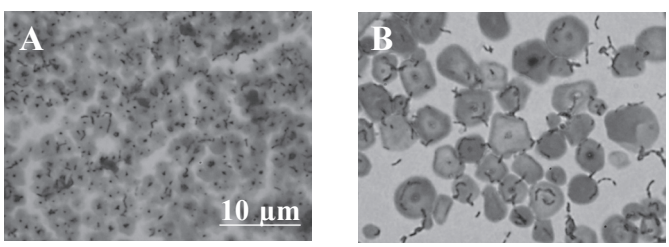

Figure 7. Binding of cells of *L. amylovorus* to glutinous rice starch (A) and corn starch (B) using Gram staining and observing under a light microscope (100x magnification).

DISCUSSION AND CONCLUSION

Some strains of *Lactobacillus* and *Bifidobacterium* spp. were reported to grow and produce the large clear zone on the corn starch agar plates, implying that the starch-degrading enzymes were induced in the presence of starch in the culture media (Nakamura, 1981; Wang et al., 1999; and Crittenden et al., 2001).

Lactobacillus amylovorus is one of the major amylolytic lactic acid bacteria (ALAB) that can ferment raw materials such as corn starch (Nakamura, 1981) due to the ability of their α-amylases to partially hydrolyze raw starch (Rodriguez et al., 2000). As native starch is cheaper and easier to obtain than glucose, its use as a carbon source in combination with *L. amylovorus* offers potential for development as a nutraceutical product. This study investigated the hydrolysis and adhesion to GS, CS, PS, and MS.

The crystalline structure of starch can be altered by physical, chemical, and biochemical modification. Heating starch in the presence of excess water results in the loss of birefringence because of the melting of starch crystallites, and is accompanied by rapid swelling of the granule (Xie et al., 2006). It was necessary, therefore, to examine the effect of increased temperature on the loss of birefringence of starch granules.

The process of preparing the culture medium with 2.0% (w/v) in preparation of starch agar plates at 47±2°C after autoclaving did not change the granular structure of the native starches. The granules remained intact with birefringence and did not collapse or disrupt, although the loss of maltese cross patterns in some granules was observed. These results were in agreement with Yotsawimonwat et al. (2007), showing that tapioca starch exhibited birefringence while heating at 47.3±1.15°C. However, in his case, when the temperature was increased to 62.0±1.73°C, some starch granules lost birefringence.

Lactobacillus amylovorus grew on all of the different starch agar plates. However, it was only able to produce a clear zone around the bacterial colonies on the GS and CS agar plates. *L. amylovorus*, however, was not able to hydrolyze all of the types of starches used in this experiment. This result might imply that *L. amylovorus* produces an extracellular amylase (Nakamura, 1981) specific to hydrolyzing only some types of starches. This study found that *L. amylovorus* was able to use native starch as a carbon source, instead of glucose; and could hydrolyze starch granules. This hydrolysis ability was also confirmed in the study using broth medium containing the same formula as the agar plates. The broth medium study was used to evaluate the morphology change of starch granules together with the pH change after incubation for five consecutive days. The culture pH dropped rapidly from about 7.00 to 4.00 ($p < 0.05$) from day 0 to day 5 in all substrates. The results demonstrated that *L. amylovorus* could convert the starch into lactic acid (Reddy et al., 2008). Additionally, scanning electron micrograph evidence showed a significant difference ($p < 0.05$) between days 0 and 5 in the granular structure of the starch incubated in the broth medium with bacteria. A porous network was seen in both GS and CS granules compared to the control at day 0. It would appear that the bacterial enzyme hydrolyzes the starch granules from the outer surface to the inner layer. This observation was supported by Wang et al. (1999) who reported that digested granules of starch were colonized by *L. amylovorus* cells.

Adhesion of *L. amylovorus* to various types of starch granules was tested to see the correlation to starch hydrolysis. The number of *L. amylovorus* cells bound to various types of starch granules in PBS pH 7.0 increased proportion-

ally with the concentration of starch from 0.5-2.0% (w/v). A moderate adhesion of bacterial to starch granules was detected in the suspensions of PS and MS, while high adhesion was observed when GS and CS were used, reaching approximately 90% adhesion. Prior studies also found increasing numbers of cells of *L. amylovorus* binding to an insoluble corn starch when the concentration of either starch or amylose in the starch was increased (Imam and Kura 1991; Tarahomjoo et al., 2008). The differences in the binding capacity of the *L. amylovorus* cells to starch granules might, in part, be due to the resulting substrate having significantly different surface morphology (Imam and Kura, 1991). Gancz et al., (2005) suggested that plant source, chemical compositions, and other physiochemical properties play an important role in determining the adhesion of bacteria. The binding capacity of bacteria to starch granules was confirmed by Gram staining and viewed under a light microscope. Results demonstrated that the binding of *L. amylovorus* cells to starch granules was associated with the percentage adhesion in starch suspension.

In conclusion, while *L. amylovorus* did not adhere well to all types of native starch granules, there was a correlation between the high adhesion of starch granules and starch hydrolysis in GS and CS. Consequently, native starch, particularly GS and CS, might be developed as a nutraceutical product or in combination with a probiotic that might enhance the beneficial effect for colonic bacteria.

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