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Selection of lactic acid bacteria for exopolysaccharide production

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ABSTRACT

An important source of natural alternative to commercial additives that are commonly extracted from plants and animals is the exopolysaccharide (EPS) produced by lactic acid bacteria (LAB). A screening for EPS production *by Lactobacillus delbrueckii*, *Lactobacillus rhamnosus* NBRC 3425 and *Weisella paramesenteroides* was conducted to identify which among these three LAB would produce the highest yield of EPS. The test organisms were grown in a Semi-defined Medium (SDM) of Sanchez et al (2006) with some modifications. EPS production was confirmed by the formation of precipitate after mixing the broth medium with 95% absolute ethanol. Results of total sugar analysis by phenol-sulfuric acid assay revealed that estimated EPS yield of *L. rhamnosus* NBRC 3425 was significantly higher at $p<0.05$ than those of *W. paramesenteroides* and *L. delbrueckii* ssp. *lactis* with values of 0.1355g/L, 0.0652g/L and 0.0544g/L, respectively even though their viable count did not differ significantly from each other. Correspondingly, the pH of *L. rhamnosus* NBRC 3425 media was also significantly higher (pH 4.03) than *L. delbrueckii* (pH 3.60) and *W. paramesenteroides* (pH 3.83).

Keywords: exopolysaccharide, lactic acid bacteria, phenol-sulfuric acid assay, Semi-defined medium (SDM), total sugar

INTRODUCTION

The global market for hydrocolloids which includes many polysaccharides, is still dominated by plant and algal polysaccharides (eg, starch, galactomannans, pectin, carrageenan and alginate) (Imeson 2010). The animal derived proteinaceous hydrocolloids gelatin and casein are also utilized. The functional properties in foods of these polymers are determined by subtle structural characteristics. However, these polysaccharides may not always be readily available in the quality needed or their rheological properties may not exactly match

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those required. Most of the plant carbohydrates used are chemically modified to improve their structure and rheological properties (De Vuyst & Degeest 1999, Roller & Dea 1992, Tombs & Harding 1998). Their use is hence strongly restricted. For instance, the European Union (EU) allows their addition only in some food products such as bakery products (Abbas et al 2010, De Vuyst & Degeest 1999).

An alternative class of biothickeners are microbial exopolysaccharides (EPS) (De Vuyst & Degeest 1999). Bacterial exopolysaccharides (EPS) show great diversity and functions, and their production is not limited by taxa (Nwodo et al 2012). A number of Lactic Acid Bacteria (LAB) can produce a variety of long chain sugar polymers, called EPS which are mainly employed for the production of fermented dairy products. Most LAB-producing EPS belong to the genera *Streptococ*cus, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Pediococcus* (Florou-Paneri et al 2013). They are synthesized either extracellularly from sucrose by glycansucrases or intracellularly by glycosyltransferases from sugar nucleotide precursors (Florou-Paneri et al 2013, Ganzle & Schwab 2009).

Sutherland (1990) found that ESPs are used because of their capacity to control the texture of foods and to prevent or reduce ice crystal formation in frozen foods. They may also influence the appearance and color as well as the flavor of prepared foodstuff. Bacterial cellulose is preferred over plant cellulose as it can be obtained in higher purity and exhibits a higher degree of polymerization and crystallinity index. It also has higher tensile strength and water holding capacity than that of plant cellulose (Chawla et al 2009, Shoda & Sugano 2005). To date, EPS produced by LAB have received increasing interest mainly because of their GRAS (Generally Regarded as Safe) status (Sutherland 1998). The GRAS and probiotic status of some lactic acid bacteria could provide impetus for more preference for their consumable EPS products (Badel et al 2011).

The greatest potential of bacterial EPS is related to their use in high-value market niches, such as cosmetics, pharmaceuticals, food ingredient, functional foods and biomedicine, in which traditional polymers fail to comply with the required degree of purity or lack some specific functional properties (Freitas et al 2011). Therefore, the search for bacteria with high ESP and ESP conjugate (bioflocculants) yields is an ongoing process (Nwodo et al 2012, Mabinya et al 2012). This study aimed to select among three strains of lactic acid bacteria capable of producing higher yield of exopolysaccharide and evaluate possible factor/s affecting production of the biopolymer.

MATERIALS AND METHODS

Lactic Acid Bacteria Isolates

Pure cultures of *Lactobacillus delbrueckii* ssp. *lactis* and *Weisella paramesenteroides* were provided by the Food Microbiology Laboratory-Food Science Cluster, College of Agriculture, University of the Philippines Los Baños (UPLB), Laguna and *Lactobacillus rhamnosus* NBRC 3425 was provided by the National Institute of Molecular Biology and Biotechnology (BIOTECH), UPLB, Laguna, Philippines. The stock culture was maintained in MRS broth by monthly transfer and storage at 4°C.

Culture Media for Exopolysaccharide Production

The Semi-defined Medium (SDM) of Sanchez et al (2006) with some modifications was used to grow the test organisms for the production of exopolysaccharide with the following composition (in grams per liter). Dextrose, 20 (carbon source); Tween 80, 1; ammonium citrate, 2; sodium acetate, 5; $MgSO_4.7H_2O$, 0.1; $MnSO_4$, 0.05; K_2HPO_4, 2; Casamino Acid, 5 (instead of Yeast Nitrogen Base); Tryptone, 10. Fifty (50) ml of the growth medium was dispensed to individual 250ml Erlenmeyer flask prior to sterilization for 15min at 121°C.

Fermentation Conditions for Screening

The bacterial isolates were screened for their ability to produce EPS by inoculating active cultures in a sterile 50ml modified Semi-Defined Medium (SDM). Each flask was inoculated with 2% 10^8 CFU/ml of 16-18 hour old inocula and incubated at their respective optimum temperature for growth [*Weisella paramesenteroides (*30°C), *Lactobacillus delbrueckii* ssp. *lactis* and *Lactobacillus rhamnosus* NBRC 3425 *(*37°C)] for 48 hours. The medium contained 2% (wt/vol) glucose as the carbon source, with an initial pH of 6.2.

Extraction of Exopolysaccharide

Cold absolute ethanol extraction was adopted in the extraction of EPS using the method of Rimada and Abraham (2003) with some modifications. The 48-hour old bacterial cultures in 50ml SDM broth was centrifuged (Hermle, Germany) at 6000rpm for 15min at 4°C to remove cell pellets. Then the supernatant was precipitated with double volume of 95% chilled ethanol and stored overnight at 4°C to allow precipitation of EPS. After overnight incubation, a second extraction was done as above. Finally, the mixture was re-centrifuged at 6000rpm for 15min at 4°C to collect the crude EPS. The collected EPS was freeze-dried (GT2 Leybold-Heraeus, Germany) for 6-8 hours. The total carbohydrate present in the pellet was estimated by phenol-sulphuric acid assay (Dubois et al 1956).

Test for Lactic Acid Production by LAB Culture

The test organisms were grown in Glucose Yeast Peptone Agar (GYPA) with calcium carbonate. After 18-24 hours of incubation at 37°C, colonies were observed with the formation of clear zones which is an indication of acid production by the LAB.

Viable Count Determination

Total viable count of the test organisms previously grown in MRS broth at 37°C for 16-18 hours was determined prior to inoculation (2% inoculation rate) in the modified Semi-defined Medium (SDM) for exopolysaccharide production. Determination of viable cell counts was carried out by serial dilution up to 10^{-8} in sterile 0.1% peptone water and pour plating. Plates were incubated at incubation temperatures corresponding to the requirement of the test organism for 24-48 hours to obtain sufficient growth. Viable count was reported as colony forming unit (CFU/ml).

Estimation of EPS Yield

Total polysaccharide content was measured by phenol–sulfuric acid assay using D-glucose as calibration standard (Dubois et al 1956). Equal volumes of sample and glucose standard were mixed with 5% phenol (JT Bakers, USA) in test tubes and then 2.5mL of concentrated sulfuric acid (95–97% Sigma-Aldrich, USA) was added. The solution was mixed in a gentle vortex and immediately soaked in ice water bath. After allowing the sample to cool for 30min, absorbance of the solutions was measured at 490nm using UV-VIS spectrophotometer (Shimadzu, Japan).

pH Determination of Broth after Fermentation

The pH pen was calibrated with pH 4.0 and 7.0 buffer solutions. The final pH of the 48-hour fermented medium was measured directly using a digital pH pen (Milwaukee Instruments, series no. 110774).

RESULTS AND DISCUSSION

Selection of Lactic Acid Bacteria for Exopolysaccharide Production

In this study, the selection of EPS-producing LAB was based on published literatures. This is more focused on the selection of the LAB strain with good EPS producing ability. Cultures of *Lactobacillus delbrueckii* ssp. *lactis*, *Lactobacillus rhamnosus* NBRC 3425 and *Weisella paramesenteroides* were plated on Glucose Yeast Peptone Agar with calcium carbonate (Figure 1). Growth on the solid medium of the three *Lactobacillus* strains showed clear zones of all colonies which means that there is production of lactic acid typical of LAB.

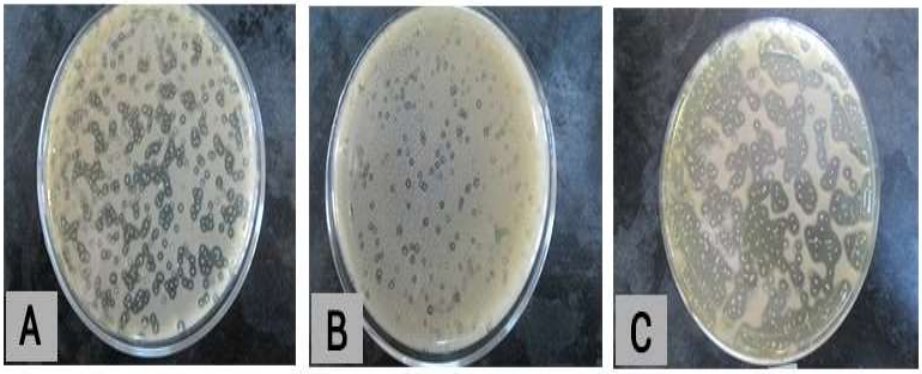

Figure 1. Lactic acid bacteria culture showing clear zones in GYPA with 5% calcium carbonate (a) *Lactobacillus delbrueckii* ssp. lactis (b) *Weisella paramesenteroides* (c) *Lactobacillus rhamnosus* NBRC 3425

Estimated Exopolysaccharide Production

Test organisms were grown in broth medium (modified SDM) with 20% glucose as the carbon source with an initial pH of 6.2 and incubation temperature of

37°C (*L. rhamnosus* NBRC 3425 and *L. delbrueckii* ssp. *lactis*) and 30°C (*W. paramesenteroides)*. The Phenol-sulfuric acid assay revealed that the estimated EPS yield of *L. rhamnosus* NBRC 3425 was significantly different at p≤0.05 than those of *W. paramesenteroides* and *L. delbrueckii* ssp. *lactis* with values of 0.1355g/L, 0.0652g/L and 0.0544g/L, respectively (Table 1). Different strains of bacteria have varying capacities to synthesize exopolysaccharide regardless of the medium, availability of nutrient, temperature, pH, etc., where they are cultivated. Most bacteria produce EPS under all conditions, but the quantities and the composition of EPS are strain-dependent and affected by the nutritional and environmental conditions (Looijesteijn et al 1999). Biosynthesis of EPS differs among genera and is an energy-dependent process (Patel et al 2010).

Table 1.Mean viable count (cfu/ml), EPS production (g/L) of test organisms and final pH of modified Semi-Defined Medium

Test Organism	Parameters		
	Final pH	EPS Production(g/L)	Viable Count (cfu/ml)^{ns}
W. paramesenteroides	3.83^{b}	0.0652^{b}	1.98 x10^{9}
L. delbrueckii ssp. *lactis*	3.60^{c}	0.0544^{b}	2.63 x10^{9}
L. rhamnosus NBRC 3425	4.03^{a}	0.1355^{a}	2.26 x10^{9}

The mean difference is significant at p≤0.05

Results agree with the findings of Macedo et al (2002) as reported by Tsuda (2013) where *Lactobacillus rhamnosus* RW-9595M exhibited the highest recorded yields of hetero-EPS (2.78g/L). In another study of Bergmaier et al (2003) a very high EPS concentration of 2.3g/L (2300mg/L) was obtained during pH-controlled batch cultures of *Lactobacillus rhamnosus* RW-9595M in 8% whey permeate medium supplemented with $MgSO_4.7H_2O$, $MnSO_4.H_2O$, Tween 80, and yeast extract for final concentrations of 0.5g/L, 0.05g/L, 1mL/L, and 10g/L, respectively.

Relationship of Resulting pH with EPS Yield

After 48 hours of incubation, the final pH of modified SDM decreased from 6.2 to 3.6 (*L. delbrueckii* ssp. *lactis*), 3.83 (*W. paramesenteroides*) and 4.03 (*L. rhamnosus* NBRC 3425). It was observed that the medium exhibiting the least change in pH during fermentation produced the highest EPS yield. This decrease in pH during fermentative production is caused by the accumulation of gluconic, acetic or lactic acids in the culture broth (Kongruang 2008). The pH which is an important factor affecting EPS yield was significantly lower (p≤0.05) in *L. delbrueckii* ssp. *lactis,* compared to *W. paramesenteroides* and *L. rhamnosus*

NBRC 3425 (Table 1). The decrease of the medium's final pH correspondingly resulted in a significant decrease in EPS yield, because when acidification occurs resulting to lactate production, enzyme glycohydrolases are activated approximately at pH 5 causing degradation of the EPS. The possible presence of glycohydrolases in the fermentation medium hydrolyzes EPS to monomers, causing such a decrease in EPS yield during prolonged fermentation (Yang et al 2010).

Relationship of Growth and EPS Yield

It has been reported that biosynthesis of EPS is growth-associated. Results of this study however, revealed that although there was no significant difference observed in the viable count of the test organisms a significant difference was noted in the EPS production of *L. rhamnosus* NBRC 3425 ($p \leq 0.05$) from *W. paramesenteroides* and *L. delbrueckii* ssp. *lactis*. This could possibly imply that *L. rhamnosus* NBRC 3425 is a very good producer of EPS or it may not always be true that EPS production is growth-associated, although this claim still needs further elucidation. Pham et al (2000) reported that Manca de Nadra et al (1985) and Kojic et al (1992) observed both growth-associated and non-growth associated production kinetics. Gassem et al (1995) also found that there was no association between growth rate or acid production and polysaccharide production in different media by LAB (strains CH15, YB57, and YB58 and *S. salivarius* subsp. *thermophilus* ST3). Recently, Looijesteijn et al (1999) indicated an uncoupling of growth and EPS production when the production of EPS by *L. lactis* subsp. *cremoris* NIZO B40 was investigated. According to them, a possible explanation for this uncoupling is the fact that optimal conditions for EPS production and growth are not the same (Pham et al 2000).

CONCLUSION

The production of bacterial exopolysaccharide is directly affected by the pH of the medium where it is grown since lower pH favors degradation of the polysaccharide. Results of the present study revealed that *L. rhamnosus* NBRC 3425 produced a significant amount of EPS compared to *W. paramesenteroides* and *L. delbrueckii* ssp. *lactis* even if the viable count does not significantly vary from the two test organisms.

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