Biosci. Biotechnol. Biochem., **74** (2), 423–426, 2010

Note

Solid State Fermentation for Extracellular Polysaccharide Production by *Lactobacillus confusus* with Coconut Water and Sugar Cane Juice as Renewable Wastes

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Received September 8, 2009; Accepted October 26, 2009; Online Publication, February 7, 2010
[doi:10.1271/bbb.90663]

Extracellular polysaccharide (EPS) production by *Lactobacillus confusus* in liquid and solid state fermentation was carried out using coconut water and sugarcane juice as renewable wastes. High concentrations of EPS of 62 (sugarcane juice) and 18 g/l of coconut water were produced in solid state fermentation when nitrogen sources were reduced 5-fold from the original medium.

Key words: solid state fermentation; extrapolysaccharide (EPS); *Lactobacillus confuses*; coconut water; sugarcane juice

Recently, the demand for natural bio-polymers for application in the food industry as thickeners, stabilizers, emulsifiers, binders, gelling agents, and film formers has increased since they are generally recognized, as safe materials. Extracellular polysaccharides (EPS) are biopolymers produced by many microorganisms. Lactic acid bacteria are recognized as relatively safe microorganisms, and EPS production by lactic acid bacteria such as dextran and fructan has been studied,[1–5] although there are some pathogenic lactic acid bacteria. Especially, *Lactobacillus confusus* can produce large amounts of dextran, which has medical applications, as a blood plasma extender, a blood flow improvement agent, and a cholesterol lowering agent, also plays a role in separation technology and can even function as a microcarrier in tissue culture.[6]

Most lactic acid bacteria are fastidious microorganisms and require many growth factors for bioactivity, such as yeast extract, beef extract, and peptone, which are expensive nutrients. EPS production by lactic acid bacteria usually requires expensive nutrients for higher growth and production of EPS. Cheaper alternative substrates might substitute, for these to reduce production costs.

Thailand produces waste coconut water at more than 200,000 tons a year, most of which is discharged after dilution with water, which causes environmental pollution.[7] It has been reported to be a low-cost carbon source for EPS production by *Agrobacterium* sp.[8] and by *Sclerotium rolfsii* MTCC2156.[9] An alternative carbon source is sugar-cane juice, is also a waste material, in local areas and also an inexpensive medium source that has a high sucrose content (7–14%).[10]

During the course of EPS production by submerged fermentation, high viscosity and shear stress have become problems in mixing, heat transfer, nutrient transfer, and other rheological behavior during lactic acid bacteria cultivation. As an alternative, solid state fermentation has been suggested to solve the problems of rheological fluidic behavior in EPS production such as that of xanthan and succcinoglucan.[11–13] Solid state fermentation has some advantages, including improve the product yield, easier product recovery, and reduced energy requirement.

This study carried out on a laboratory scale, and focused on practical production of EPS by *Lactobacillus confusus* TISTR1499 from coconut water and sugar-cane juice, used as recycled organic wastes, and medium optimization with the combination of coconut water and sugarcane juice. Coconut water and sugar-cane juice were subjected to solid state fermentation to enhance EPS production.

Lactobacillus confusus TISTR 1499 isolated from traditional northern Thailand fermented pork (Nham) was used. The strain was deposited for culture collection at the Thai Institute of Scientific and Technological Research (TISTR).

Modified MRS medium, which is frequently used in *Lactobacillus* sp. culture, was used in this study.[14]

The modified MRS medium used here consisted of (g/l): peptone 5.0, beef extract 2.5, yeast extract 2.5, sucrose 20, K_2HPO_4 2.0, di-ammonium hydrogen citrate 2.0, $CH_3COONa{\cdot}H_2O$ 7.6, $MgSO_4$ 0.1, $MnSO_4{\cdot}7H_2O$ 0.4, and Tween 80, which was also added (1 ml). A combination of mature coconut water and sugar-cane juice was used in place of water to prepare the MRS medium (initial total sugar content in MRS media with coconut water at 20, 28, 36, 48, 52, and 60 g/l and media with sugar-cane juice at 20, 36, 52, 68, 84, and 100 g/l). Total sugar and EPS were assayed as a control treatment in fresh medium broth without bacterial inoculation, and all EPS concentrations are reported with these subtractions. Coconut water contained approximately (g/l): total solid 40, total carbohydrate 17.8 (glucose 5, fructose 6.1, and sucrose 6.7), organic acid 4.5, fatty acid 6.6, trace elements (Mn 0.19, Mg 0.046, K 0.41, Na 0.23), and N as a minor component at 10 mg/l. Sugar-cane juice used here contained approximately

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100 g/l of total sugar and 1.6 g/l of protein. In addition, amino acid and vitamins were also present as growth factors of lactic acid bacteria.[15)] The medium was adjusted to pH 7.0 before autoclaving (121 °C, 15 min).

In order to prepare an inoculum, the frozen culture was rejuvenated in 10 ml of modified MRS medium for 24 h at 35 °C. The OD_{650} was 0.8 for the preculture. Liquid culture was prepared in 250-ml Erlenmeyer flasks containing 100 ml of liquid maintained at pH 7.0 with 10% inoculum for 24 h at 35 °C. For solid state fermentation, 20 ml of medium containing 2% agar was poured into a sterilized petri dish (0.9 cm in diameter) and allowed to cool until it solidified. It was incubated at 55 °C for 6 h to produce a dry surface. Then 1 ml of inoculum was spread onto the agar surface, and this was cultivated for 24 h at 35 °C under a humidified atmosphere in static mode. In order to harvest cells and EPS from the agar surface, the surface was washed 2 times with 50 ml of distilled water until all the cells and EPS were made into a suspension condition.

The cell mass concentration was determined by OD_{650} and calculation for dry cell weight by a standard curve. When solid state fermentation was carried out, biomass dry weight was evaluated in the suspended liquid with OD_{650} after it was washed with distilled water 2 times from the agar surface, and the total dry weight of the cells was calculated based on the agar medium(0.02 liter) in the petri dish.

EPS was determined by the method of Duenas *et al.*[16)] using trichloroacetic acid at a final concentration of 30% w/v to remove residual proteins. The supernatant (100 ml) was stored at 4 °C for 30 min, followed by cool ethanol precipitation (part of supernatant to 3 parts of 80% cool ethanol). The supernatant from this step was analyzed for residual total sugar content. Then the crude cool ethanol EPS part was dissolved with the same amount of distilled water and precipitated with cool ethanol. Precipitation was repeated 3 times and the precipitation was dried to constant weight at 55 °C. Total sugar in the medium was determined by the conventional phenol sulfuric acid method using glucose as standard. The data in Fig. 1 to Fig. 3 are from triplet experiments.

Coconut water and sugar-cane juice contain large amounts of sucrose. The C/N ratio appears to be important for EPS production.[9,10)] The effect of the addition of coconut water and sugar-cane juice to the modified MRS medium was investigated in liquid fermentation. As shown in Fig. 1, the highest cell mass yield, of 2.5 g/l, was obtained without the addition of any coconut water, but EPS production was low. With the addition of 100% coconut water, EPS production was high, at 12.9 g/l, with a relatively low cell mass concentration of 1.3 g/l. In these cultures, cell mass growth reached a stationary stage after about 12 h, but EPS production continued increase and attained the maximum level after 20–24 h (data not shown). EPS productivity increased dramatically following the increase in the total sugar content of the medium.

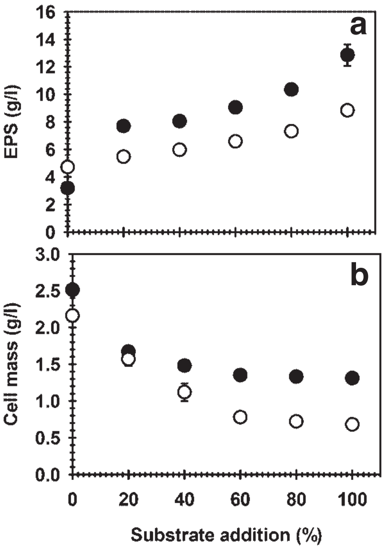

Fig. 1. Effects of Initial Coconut Water (●) and Sugar-Cane Juice (○) Addition in Place of Dilution by Water (0–100%) in Modified MRS Medium on EPS (a) and Cell Mass (b) Production by Liquid Fermentation of *Lactobacillus confusus*.

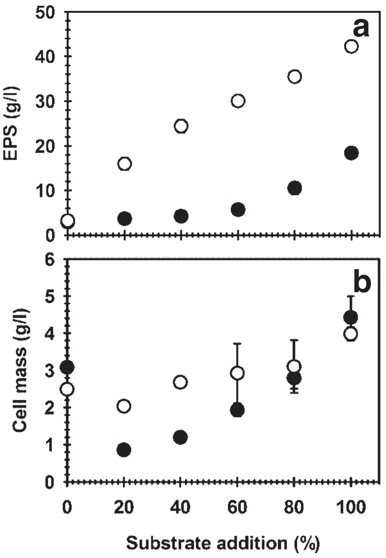

Fig. 2. Effects of Initial Coconut Water (●) and Sugar-Cane Juice (○) Addition in Place of Dilution by Water (0–100%) in Modified MRS Medium on EPS (a) and Cell Mass (b) Production by Solid State Fermentation of *Lactobacillus confusus*.

As shown in the lower half of Fig. 1, experiments to assess the effects of the addition of sugar-cane juice on EPS and cell mass production were carried out. The results were similar to the ones obtained for coconut water. Sugar-cane juice (100%) produced only 8.8 g/l of EPS and 0.7 g/l of dry cell mass despite the high sugar content of the medium. Production was lower than that obtained by the addition of coconut water. Large amounts of total sugar remained in the medium (83.9 g/l). With the addition of sugar-cane juice, the pH of the medium to decreased to acidic conditions during culture (about pH 4.5), which probably suppressed cell growth and enzyme activity for EPS production.[11,12)]

On the other hand, solid state fermentation with the addition of various concentrations of coconut water and sugar-cane juice was carried out. As shown in Fig. 2, surprisingly, cell mass and EPS production increased in both types of solid state fermentation. With the addition of 100% coconut water, 18.4 g/l of EPS and 4.4 g/l of dry cell mass were obtained on agar fermentation, higher

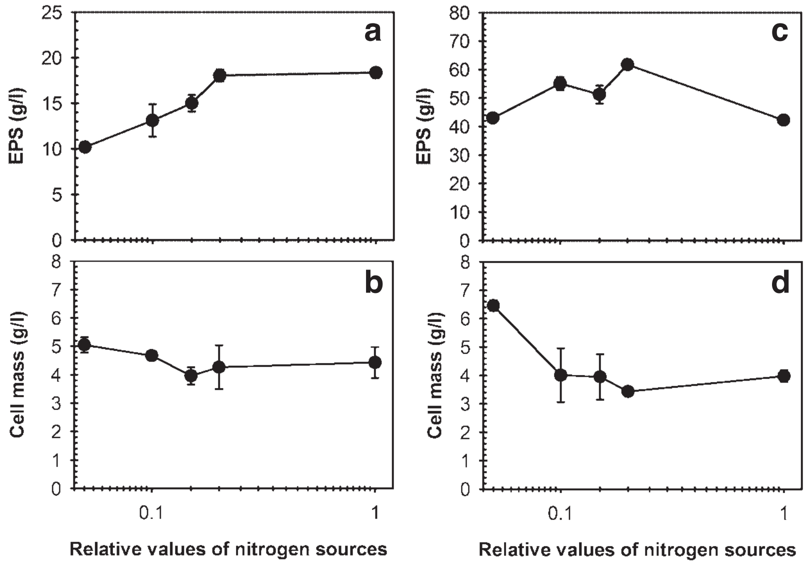

Fig. 3. Effects of Nitrogen Source Concentration on the Productions of Extracellular Polysaccharide (EPS) (a) and Cell Mass (b) in 100% of Coconut Water with 60 g/l of Total Initial Sugar Content and EPS (c) and Cell Mass (d) in 100% of Sugar-Cane Juice with 100 g/l of Total Initial Sugar Content by *Lactobacillus confusus* in Solid State Fermentation (SSF).

The relative value of the nitrogen source shows the addition of 1 × PYE (=5 g/l, Peptone, 2.5 g/l, Yeast Extract, and 2.5 g/l, Beef Extract), 0.2 × PYE, 0.15 × PYE, 0.1 × PYE, and 0.05 × PYE.

than those obtained on liquid fermentation (Fig. 1). It should be emphasized that cell mass and EPS production in sugar-cane juice increased to 4.0 and to 42.2 g/l, respectively, and that EPS concentration was 5 times greater than those obtained in liquid fermentation. Such high concentrations of EPS production has not been reported to date. This seemed impossible, due to the viscosity or the problem of transfer in liquid fermentation, even though the addition of sugars and nutrients was carried out carefully in submerged culture under pH control. There was an attempt at EPS production by lactic acid bacteria on agar medium in a screening step with a relatively high sugar concentration (100 g/l), but the total amount of EPS was not determined. This might be the first report that a high sugar concentration was used in solid state fermentation and a large amount of EPS was obtained without pH control.

In liquid medium fermentation, there have been many reports stating that the initial sugar concentration affected the EPS yield.[17,18)] For instance, *Lactobacillus reuteri* strain LB 121 produced the highest level of EPS (over 10 g EPS/l) with an excess carbon source and a limited nitrogen source.[2)] High energy generation with low nitrogen-led low protein synthesis resulted in sugar molecules, and energy was transferred to EPS synthesis.

In solid state fermentation, high cell mass and high EPS production were obtained. It is suggested that high energy generation with the high sugar concentration and the relatively low and moderate amount of nitrogen (low C/N ratio) of sugar-cane juice resulted high EPS production and relatively high cell-mass production. In addition, it is suggested that EPS capsules might functions as a biofilm and protect cells from toxic chemical compounds and stressful environmental factors such as pH decrease.[12,13)]

The nitrogen sources used in this study, including peptone, yeast extract and beef extract, are expensive. Therefore, reduction in the amount of the nitrogen in solid state fermentation was tried for EPS and biomass production. As shown in Fig. 3a, a reduction in the quantities of the sources of nitrogen resulted in decreases in EPS production, from 18.1 to 10.2 g/l at 0.05 × PYE in coconut water. However, in sugar-cane juice, EPS production increased, with a decrease in the quantities of the source of nitrogen (Fig. 3c). The maximum EPS yield was 61.8 g/l when a 5-fold reduction of the nitrogen source was used (0.2 × PYE, that is, 1.0 g/l of peptone, 0.5 g/l of yeast extract and 0.5 g/l of beef extract). It is an advantage that pH control was not carried out in this fermentation, but EPS production proceeded from 8.8 g/l of liquid medium (Fig. 2) to 61.8 g/l, which is about a 7-fold increase at a 5-fold reduction in the nitrogen source (0.2 × PYE). In the coconut water experiment (Fig. 3a), a relatively high amount of EPS, 18.1 g/l, was produced with a 5-fold reduction in the nitrogen source as compared with 12.9 g/l of EPS production in liquid medium. This solid state fermentation are enhancement of EPS production by *Lactobacillus confusus* TISTR 1499 using coconut water and using sugar-cane juice. It might become a practical technology for EPS production with recycled use of waste coconut water and sugar-cane juice.

Acknowledgments

This work was supported by the JSPS-NRCT core university program. The authors also gratefully acknowledge research funding from the Thai Commision on Higher Education of the Ministry of Education.

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