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*Original Article*

## Exopolysaccharide production by *Lactobacillus confusus* TISTR 1498 using coconut water as an alternative carbon source: the effect of peptone, yeast extract and beef extract

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### Abstract

Coconut water (CW) is a by-product of food industry and has little value in Thailand. It is usually discarded as a waste into the environment. Consequently, we developed a value added process of exopolysaccharide (EPS) production using *Lactobacillus confusus* TISTR 1498 and coconut water. The effect of three expensive supplements (peptone, yeast extract and beef extract) on EPS and biomass production was investigated at 35°C for 24 h. Using a mod-MRS-CW medium, prepared by replacing the de-ionized water with 100% CW and supplemented with 20 g/l crystalline sucrose and a reduced quantity (50%) of the three expensive supplements (5 g/l of peptone, 2.5 g/l of yeast extract, and 2.5 g/l of beef extract) gave the highest yield of EPS (12.3 g/l). By optimizing the conditions for fermentation (pH 5.5, agitation speed at 50 rpm and initial sucrose concentration of 100 g/l), EPS yield increased up to 38.2 g/l. When compared with the modified MRS medium, the medium supplemented with CW was found to be suitable for the reduction of cost spent on the organic nitrogen and growth factors (savings close to 50%).

**Keywords:** coconut water, fermentation, exopolysaccharide, batch culture, lactic acid bacteria, *Lactobacillus confusus*

### 1. Introduction

Exopolysaccharide (EPS) is a biopolymer produced by many microorganisms (Kumar *et al.*, 2007). In recent years, increasing demand for natural polymers for their application in food industry as thickeners, stabilizers, emulsifiers, binders, gelling agents, and film former have led to an interest in microbial EPS (Sutherland and Kennedy, 1996; De Vuyst and Degeest, 1999). Especially, the microorganisms of GRAS (Generally Recognized as Safe) status, such as lactic acid bacteria (LAB), have gained increasing attention. In the past

decade, studies on EPS production by LAB have reported on factors affecting the production, properties of EPS, structure, synthesis mechanism, gene regulation, metabolic pathways and modification of metabolic flux (Levander *et al.*, 2002). The majority of EPSs are heteropolysaccharides (HePSs) containing long chain repeating subunits of two or more monosaccharides. They are typically produced in small amounts up to 2 g/l. Homopolysaccharides (HoPSs) are a minority, but they can be generated in large quantities suiting commercial needs, such as dextran by *Leuconostoc mesenteroides*, *Streptococcus mutan*, *Gluconobacter oxydan*; Levan by *Lactobacillus reuteri* strain 121; and fructan by *Lactobacillus sanfranciscensis* LTH2590 (van Geel-Schutten *et al.*, 1998; De Vuyst and Degeest, 1999; van Hijum *et al.*, 2001; Korakli *et al.*, 2002; Naessens *et al.*, 2005). With respect to these studies, the cost of fermentation plays an important

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role and when the process is scaled up to the economic scale, the production yield of EPS should be greater than 10 g/l (De Vuyst and Degeest, 1999). Several strains of LAB have been studied for EPS production. However, little is known about EPS production by *Lactobacillus confusus*, although it can produce a high amount of dextran, which is being used in medical application as blood plasma extender, a blood flow improving agent, a cholesterol lowering agent, with separation technologies or even as a micro-carrier in tissue culture (Sharpe *et al.*, 1972; De Vuyst *et al.*, 2001).

Most LAB are fastidious microorganisms, they require many growth factors to support their bioactivity. Yeast extract, beef extract and peptone, which are widely used for the cultivation of bacteria, are expensive nutrients. There are several reports about the improvement of EPS production and biomass yield by optimization of medium and culture conditions, as the yield of EPS depends on the composition of the medium and the growth conditions (Kimmel *et al.*, 1998; Degeest and De Vuyst, 1999; Degeest *et al.*, 2001; Broadbent *et al.*, 2003; Velasco *et al.*, 2006). However, most of them emphasized using expensive complex nitrogen. In the economic analyses, the cost of peptone, yeast extract and beef extract was estimated to be over 30% to the total production cost (Li *et al.*, 2006; Venus, 2006). An investigation into cheaper alternative substrates to substitute for those high cost materials is important.

Thailand produces large volumes of coconut water (CW) as a waste from the coconut processing industry and most of it is discarded into the drain. The amount of coconut water in Thailand was reported to be nearly 200,000 tons per year, and the amount is rising yearly due to the increasing number of products made with coconut milk as an ingredient for export (Unagul *et al.*, 2007).

Mature coconut water is a free byproduct and is a rich substrate for microbial cultivation (Shivakumar and Vijayendra, 2006) containing high sugar concentrations (glucose 5 g/l, fructose 6.1 g/l and sucrose 6.7 g/l), trace elements and nitrogen as a minor component approximately 10 mgN/l (Unagul *et al.*, 2007). The use of CW as a low cost carbon source for EPS production by *Agrobacterium* sp. (Shivakumar and Vijayendra, 2006) and scleroglucan by *Sclerotium rolfsii* MTCC 2156 (Survase *et al.*, 2007) has been reported. However, the use of CW as a substituted raw material for EPS production by LAB has not yet been studied.

In order to reduce the cost of EPS production, we report on the investigation carried out with CW as a cheap substitute for carbon or a source of trace elements for EPS production by *L. confusus*. The three costly components such as peptone, yeast extract, and beef extract, in the modified MRS medium were reduced in concentration or omitted (one factor at a time) and in all cases replaced with coconut water. In addition, the effect of initial sucrose concentration on EPS production was also studied.

## 2. Material and Methods

### 2.1 Microorganism

*Lactobacillus confusus* TISTR 1498 isolated from traditional northern Thailand fermented pork (Nham) was used in this study. The strain was deposited in the culture collection at the Thailand Institute of Scientific and Technological Research (TISTR). It was maintained in the MRS medium (de Man *et al.*, 1960) plus 60 % glycerol at  $-80^{\circ}\text{C}$  until required.

### 2.2 Media

The MRS medium was used to maintain and to recover the strain from its frozen state. EPS production was investigated using the modified MRS medium containing sucrose (mod-MRS medium) instead of glucose, which is usually present in the MRS medium. The mod-MRS medium consisted of (g/l): peptone -10.0; beef extract (BE)-5; yeast extract (YE)-5; sucrose -20;  $\text{K}_2\text{HPO}_4$  -2.0; di-ammonium hydrogen citrate -2.0;  $\text{CH}_3\text{COONa}$ .  $\text{H}_2\text{O}$  -7.6;  $\text{MgSO}_4$  -0.1;  $\text{MnSO}_4$  -0.4, and Tween 80 was added (1 ml/l). During the study, mod-MRS medium with mature coconut water (mod-MRS sucrose-CW) was used for the EPS production. It contained all the previously mentioned components, but the de-ionized water was replaced by 100 % mature coconut water (approximate 40 g/l of total sugar) obtained from ripened coconuts. The coconut water was collected and frozen under  $-20^{\circ}\text{C}$ . In order to adjust total sugar in the medium to the desired sugar levels, fresh coconut water was diluted or crystalline sucrose was added into the medium after thawing. The medium was neutralized by NaOH and was sterilized by autoclaving at  $121^{\circ}\text{C}$  for 15 min.

### 2.3 Inoculum preparation

To prepare the inoculum, the frozen culture was rejuvenated in 10 ml of mod-MRS medium and incubated at  $35^{\circ}\text{C}$  for 24 h. The optical density (OD = 650 nm) of the resulting suspension was adjusted to 0.8 before use.

### 2.4 Effect of omitting either the sucrose or nitrogen source supplements (peptone, yeast extract, or beef extract) on EPS production

The mod-MRS-CW medium was prepared using different concentrations of sucrose, nitrogen and other supplements as shown in Table 1. This experiment was designed to investigate the effect of these supplements on EPS production.

Table 1. Summary of modified MRS medium used in the EPS production by *Lactobacillus confusus* TISTR 1499

Formula	Sucrose (g/L)	Peptone (g/L)	Yeast extract (g/L)	Beef extract (g/L)	Coconut water (%)
1	-	-	-	-	100
2	5	-	-	-	100
3	10	-	-	-	100
4	15	-	-	-	100
5	20	-	-	-	100
6	-	2.5	-	-	100
7	-	5	-	-	100
8	-	7.5	-	-	100
9	-	10	-	-	100
10	-	7.5	1	-	100
11	-	7.5	2	-	100
12	-	7.5	3	-	100
13	-	7.5	4	-	100
14	-	7.5	5	-	100
15	-	7.5	3	1	100
16	-	7.5	3	2	100
17	-	7.5	3	3	100
18	-	7.5	3	4	100
19	-	7.5	3	5	100

### 2.5 Effect of various concentrations of three expensive components (peptone, YE and BE) on EPS production

The mod-MRS-CW medium was prepared with 0, 25, 50, 75 and 100 % reduction of peptone, yeast extract and beef extract, to study its effect of coconut water, in conjunction with these sources on EPS production.

### 2.6 Effect of coconut water level on EPS production

The mod-MRS -CW medium containing peptone 5 g/l, yeast extract 2.5 g/l and beef extract 2.5 g/l (50% reduction) was prepared by replacing de-ionized water used as a solvent for medium preparation with different amounts of ripened coconut water (0, 20, 40, 60, 80 and 100%).

### 2.7 Fermentation conditions

Batch cultivations were conducted in a 5 L fermenter (B. Braun Biostat B; Biotech International, Allentown, Pennsylvania, USA) with 3 L working volume of a test medium with a pH of 7.0 and 10 % inoculum. All the treatments were run in triplicate. EPS and biomass production were monitored for 24 h at 35°C.

### 2.8 Determination of biomass content

The absorbance of samples obtained after 24 h incubation was measured at 650 nm and the biomass concentra-

tion was calculated from a standard calibration curve.

### 2.9 Determination of EPS content

The fermented broth was centrifuged at 10,000 x g for 10 min at 4°C and the cell-free clear supernatant was used for EPS determination. Before the EPS was precipitated with ethanol, a supernatant was added with 30% (v/v) trichloroacetic acid and stored for 30 min at 4°C, to inactivate EPS degrading enzymes and to precipitate the proteins. The crude EPS was then isolated by cool ethanol precipitation (1:3 ratios). After centrifugation (3500 x g, 15 min, 4°C), the EPS pellet was dispersed in aqueous 80% ethanol and centrifuged again and this process was repeated three times. The final precipitate was dissolved in distilled water and the pellet was dried to a constant weight at 55°C (Duenas *et al.*, 2003).

### 2.10 Determination of sugars and organic acids content

The pH level was measured using a pH meter (model SA 230; Orion Research). Total sugar content was measured using phenol-sulfuric acid (Dubois *et al.*, 1956). Acetic and lactic acids, and sugars of culture broth of the heterofermentative *Lactobacillus confusus* were determined using HPLC (Shimadzu LC-10ATvp; Shimadzu Co. Ltd., Japan), under the following conditions: column- Aminex HPX-87X (300 mm × 7.8 mm), column oven temperature: 38°C (model: CTO-10 ASvp), mobile phase: 5 mM H<sub>2</sub>SO<sub>4</sub> in water with a flow rate of 0.75 ml/min, detector UV-vis (model: SPD-10Avp) at 210

nm and RI detector (model: RID-10).

### 3. Results and Discussion

#### 3.1 Effect of initial sucrose concentration on EPS production

It has been hypothesized that bacterial growth and EPS production are usually influenced by initial concentration of carbon (Wachenheim and Patterson, 1992). In the present experiment, *Lactobacillus confusus* TISTR 1498 was cultivated with different initial total sugar concentrations (20, 50, 75, 100 and 125 g/l) individually and in mod-MRS medium supplemented with three expensive components (10 g/l of peptone, 5 g/l of yeast extract, and 5 g/l of beef extract). The highest yields of EPS and biomass, in terms of cell dry weight, were 6.13 and 1.25 g/l, respectively, in the mod-MRS medium with 100 g/l total sugar. In the treatment with high initial total sugar content (125 g/l), both EPS and biomass yield dramatically decreased to 2.92 and 0.66 g/l respectively as shown in Figure 1 (A) and (B). The optimal sucrose concentration was 10% in the mod-MRS medium in concordance with research on the production of EPS by *Lactobacillus* strain LB180 (van Geel-Schutten *et al.*, 1998). High sugar content in the mod-MRS medium led to a decrease in osmotic stress which affected the yield of EPS and biomass production (Prasertsan *et al.*, 2008).

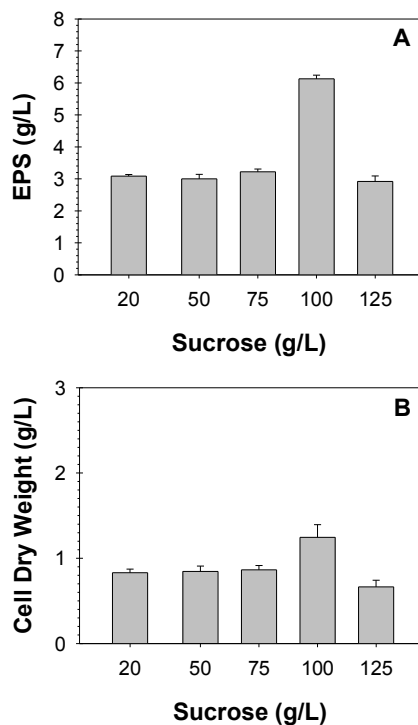


Figure 1. EPS (A) and biomass (B) yield obtained from the cultivation of *Lactobacillus confusus* TISTR 1498 under static condition at 35°C with the mod-MRS medium plus 10 g/l of peptone, 5 g/l of yeast extract and 5 g/l of beef extract.

#### 3.2 Effect of coconut water as a nutritive supplement on EPS production with mod-MRS medium

##### 3.2.1 Effect of external crystalline sucrose

The potential of coconut water for the production of EPS by the strain TISTR 1498 was determined in this experiment. De-ionized water used for the mod-MRS medium was replaced with freshly collected coconut water obtained from ripened coconuts and not supplemented with either peptone, YE or BE. Total sugar content in coconut water varied from 26.5–42.5 g/l, and mean total sugar was approximately 40 g/l. This correlated with the sucrose concentration of 2.8–4.0%, which was reported earlier (Shivakumar and Vijayendra, 2006). The effect of an external carbon source on EPS and biomass production was observed by supplementation with crystalline sucrose. It was revealed, as shown in Figure 2A and 2B, that sugar content in the coconut water is not enough to support the requirements strain TISTR 1498, hence a low yield of both EPS and biomass was obtained, 2.23 and 0.79 g/l, respectively. However, the highest yields of 3.1 g/l of EPS and 2.05 g/l of cell dry weight were obtained when 20 g/l of external crystalline sucrose was added into the mod-MRS-CW medium (approximately 60 g/l of total sugar in the medium). Although the EPS yield did not increase significantly, the higher initial total sugar concentration could induce a greater biomass by up to 120%. The external sucrose had a

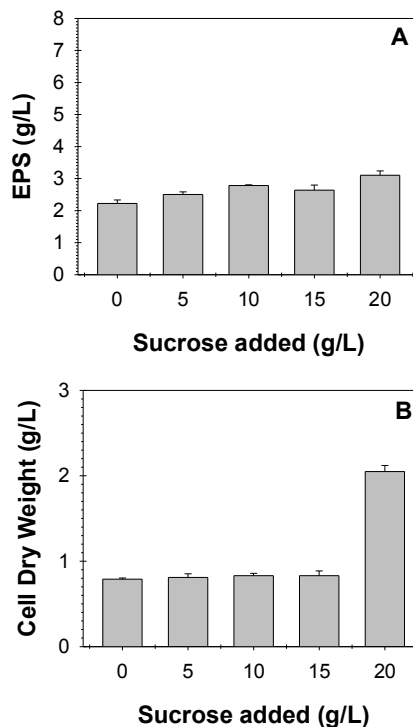


Figure 2. Effect of external crystalline sucrose on EPS (A) and biomass (B) production by *Lactobacillus confusus* TISTR 1498 in mod-MRS-CW medium plus 10 g/l of peptone, 5 g/l of yeast extract and 5 g/l of beef extract under static condition at 35°C.

significant effect on the biomass content. It was noticed that the original amount of sugar and supplements present in coconut water could not entirely support the requirements of *Lactobacillus confusus* TISTR 1498 for the EPS production. Addition of external sucrose and the three supplements (peptone, YE, and BE), sources of carbon/nitrogen, to the mod-MRS medium for high EPS production was still necessary.

### 3.2.2 Effect of organic nitrogen (peptone)

Addition of organic nitrogen (7.5 g/l of peptone) was found to influence the EPS production significantly with the highest yield of 3.7 g/l (Figure 3). The cell dry weight was in the range from 1.34-1.52 g/l when peptone was supplied from 0-10 g/l into the medium. This could be compared to experiment 1, wherein 3.1 g/l of EPS yield was obtained from 50 g/l of total sugar in mod-MRS medium with full amounts of the three supplements (peptone, YE, and BE). However, 2.95 g/l of EPS was obtained in the mod-MRS-CW medium having 2.5 g/l of peptone with no added crystalline sucrose. In other words, sugar content in coconut water (40 g/l of total sugar) and the addition of 2.5 g/l of peptone to the mod-MRS-CW medium satisfied the requirements of the strain, and the production was comparable to the mod-MRS medium. However, the EPS yield obtained from the treatment using 7.5 g/l of peptone had the highest productivity, therefore, this peptone concentration was chosen for further study in next experiment.

### 3.2.3 Effect of yeast extract

Yeast extract is not only a nitrogen source, but also a source of necessary supplements, including vitamins and minerals. The effect of yeast extract (between 1 and 5 g/l) on the EPS production was also studied (Figure 4). It revealed that the cell growth and EPS production were enhanced by adding yeast extract with a limited concentration of sugar content in coconut water. The maximum EPS production was 4.33g/l, when concentrations of peptone and yeast extract were 7.5 and 3 g/l, respectively (nearly 25 % higher than the treatment with no addition of peptone). Although the biomass yield was 1.58 g/l, it was not the highest yield. However, addition of yeast extract at 4 g/l gave the highest biomass yield of 2 g/l. Thereby, yeast extract was recognized as a good nitrogen source for LAB to enhance the EPS production. It had been shown that apart from nitrogen source, yeast extract was an essential source of  $Mn^{2+}$  and  $Mg^{2+}$  salts for lactobacilli, and the ions promoted the EPS yield through enhanced growth (Grobben *et al.*, 1998).  $Mg^{2+}$  ion associates with phosphoglucomutase enzyme, which catalyses phosphate transferring between glucose carbon C1 and C6 (Gamar-Nourani *et al.*, 1997). It is concluded that due to a lack of added vitamins, lactobacilli could not produce a high amount of EPS, and biomass and yeast extracts were advantageous over peptone as they supplied high amounts of

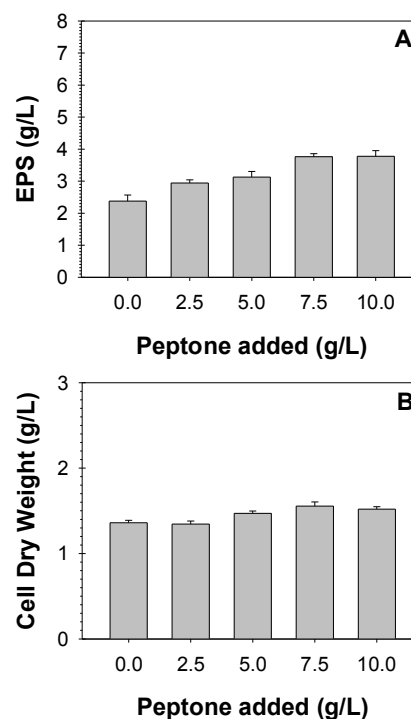


Figure 3. Effect of peptone on EPS (A) and biomass (B) production by *Lactobacillus confusus* TISTR 1498 in mod-MRS-CW medium without external crystalline sucrose, yeast extract and beef extract under static condition at 35°C.

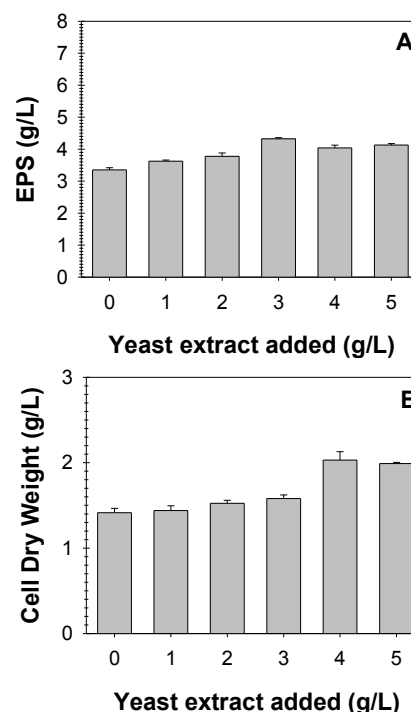


Figure 4. Effect of yeast extract on EPS (A) and biomass (B) production by *Lactobacillus confusus* TISTR 1498 in mod-MRS-CW medium plus 7.5 g/l of peptone without external crystalline sucrose, and beef extract under static condition at 35°C.

vitamins and nucleic base components (Smith *et al.*, 1973).

### 3.2.4 Effect of beef extract

Beef extract is another nitrogen source. Addition of beef extract in the range 1-5 g/l did not significantly enhance the EPS productivity as shown in Figure 5. The EPS yield was only in the range of 4.1-5 g/l, compared to that of the control (4 g/l of EPS yield). The biomass production was in a range of 1.45-2 g/l, nearly the same as the biomass content achieved in experiment 1. However, the EPS yield was lower by 20%. The result implies that high levels of nitrogen in cultivation did not affect the EPS productivity of the TISTR 1498. Similar results were described for other EPS producing strains. The EPS yield by *Pediococcus damnosus* 2.6 decreased when the C:N ratio decreased (Duenas *et al.*, 2003). Addition of poly-peptone decreased the EPS productivity of *Enterobacter cloacae* WD7 (Prasertsan *et al.*, 2008). In xanthan production, high nitrogen concentration resulted in low productivity by *Xanthomonas campestris* (Lo *et al.*, 1997). However, the effect of nitrogen source on the EPS production is still unclear and not yet fully understood (Kimmel *et al.*, 1998).

### 3.3 Production of EPS with mod-MRS -CW medium and reduction of the three supplements (peptone, yeast extract, and beef extract)

As mentioned earlier, the ratio between carbon and nitrogen influences the EPS productivity in many microorganisms. In order to increase the carbon concentration in the medium, 20 g/l of crystalline sucrose was added to the mod-MRS-CW medium. This resulted in a final total sugar content of approximately 60 g/l. The three expensive components (peptone, yeast extract, and beef extract) were added in the range of 0-100%. As shown in Figure 6, 50% reduction of the three components gave the highest yield of EPS with 12.3 g/l, but the biomass content was very low (1.75 g/l) and the highest biomass yield of 2.2 g/l was obtained at 75% strength of the three components. Interestingly, the EPS yield was nearly the same when the medium was modified by adding the three components with 0, 25 and 100% reduction. These results proved the hypothesis that increasing the nitrogen content in the medium could suppress both cell growth and EPS production.

In addition, coconut water was diluted from 0-100% and added to the mod-MRS-CW medium having 20 g/l sucrose and half strength of the three supplements (peptone, YE, and BE). The effect of coconut water concentration on EPS production was investigated. The results revealed that higher amounts of coconut water resulted in the highest EPS production to the tune of 12.3 g/l. While, no addition of coconut water to the medium resulted in increased cell productivity of 2.54 g/l with residual sugar of 1.94 g/l, but gave the lowest EPS yield of 3.1 g/l. (Table 2). Past research suggested the possibility of a low EPS yield, when high

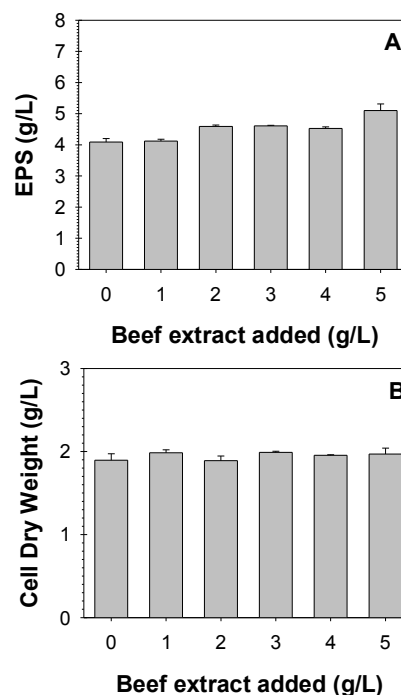


Figure 5. Effect of beef extract on EPS (A) and biomass (B) production by *Lactibacillus confusus* TISTR 1498 in mod-MRS-CW medium plus 7.5 g/l of peptone and 3 g/l of yeast extract without external crystalline sucrose under static condition at 35°C.

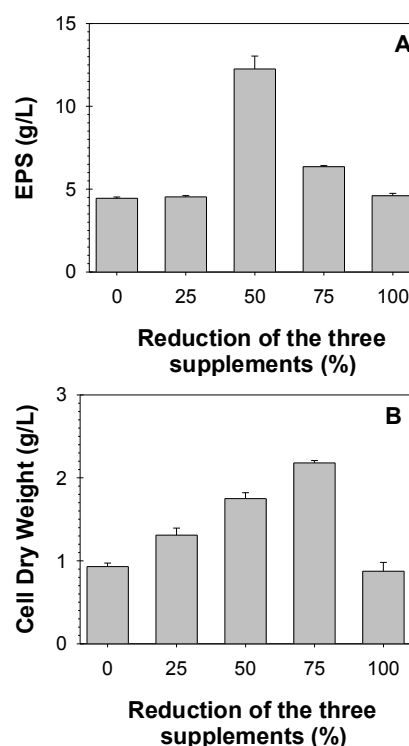


Figure 6. EPS (A) and biomass (B) yield obtained from the cultivation of *Lactibacillus confusus* TISTR 1498 under static condition at 35°C with the mod-MRS sucrose (20 g/l)-CW medium plus several percent reductions of the three supplements (peptone, yeast extract and beef extract)

Table 2. Effect of various coconut water concentrations in the mod-MRS-sucrose-CW on EPS production by *Lactobacillus confusus* TISTR 1498

Percent of coconut water added into MRS medium	EPS yield (g/L)	Cell Dry Weight (g/L)	Residual sugar (g/L)
0	3.1 <sub>a</sub>	2.54 <sub>a</sub>	1.94
20	7.9 <sub>b</sub>	1.65 <sub>b</sub>	6.15
40	8.2 <sub>b</sub>	1.47 <sub>b</sub>	15.25
60	8.9 <sub>b</sub>	1.38 <sub>b</sub>	18.20
80	10.3 <sub>b</sub>	1.36 <sub>b</sub>	21.25
100	12.3 <sub>c</sub>	1.34 <sub>b</sub>	37.10

The mod-MRS-sucrose-CW medium was contained 20 g/L sucrose, and 50% reduction of the three supplements (5 g/L peptone, 2.5 g/L yeast extract, and 2.5 g/L beef extract). The values with the same alphabet are not significantly different at the 0.05 level.

nitrogen content was added into the complex medium, which may be due to the possible generation of toxic compounds during sterilization or may be due to growth inhibiting conditions (Degeest and De Vuyst, 1999).

Based on a report of Degeest and De Vuyst (1999), the EPS production was carried out at a constant pH of 5.5,

agitation speed of 50 rpm and 100 g/l of initial sugar concentration. The high yield of EPS (38.2 g/l) and low biomass content of 1.52 g/l were obtained at 24 hours. Glucose and fructose were completely utilized at 6 and 30 hours, respectively, while at the end of fermentation, lactic acid and acetic acid production were 29.8 g/l and 7.2 g/l, respectively.

The biosynthesis of EPS needs supporting energy. A limitation of energy generation within bacterial cells during EPS formation causes a low biomass generation, because of its growth-associated formation (De Vuyst *et al.*, 2001). It also supported the phenomenon that a high C:N ratio gave a higher EPS yield with lower cell growth. It is possible that the pathway of cell growth was blocked and changed to the EPS synthesis pathway (Prasertsan *et al.*, 2008). The results obtained in the EPS production on media with high C:N ratio by the strain TISTR 1498 were similar to the results of the xanthan production (Lo *et al.*, 1997).

#### 4. Conclusions

The results from this study revealed that a cheap and naturally renewable source, such as coconut water, can be used as a substitute substrate for the EPS production by *Lactobacillus confusus* TISTR 1498. Production of this value-added product from coconut water can reduce the amount of wastewater discarded. In the present work, cultivation of the strain TISTR 1498 with coconut water alone, without any external carbon source and supplements, could not produce a high amount of EPS and biomass. In contrast, the highest EPS yield of 12.3 g/l was obtained, when it was cultivated in the mod-MRS-CW supplemented with 20 g/l crystalline sucrose and a reduced quantity (50%) of the three expensive supplements (5 g/l of peptone, 2.5 g/l of yeast extract, and 2.5 g/l of beef extract). At a constant pH of 5.5, agitation speed at 50 rpm and 100 g/l of initial sugar concentration, a high yield of EPS (38.2 g/l) and low biomass (1.52 g/l) were obtained at 24 hours. Glucose and fructose were completely utilized at 6 and 30 hours, respectively.

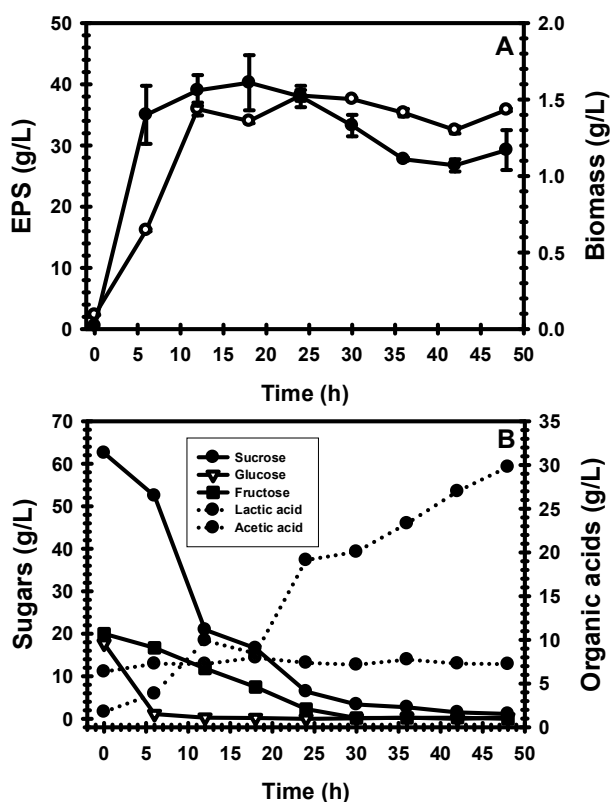


Figure 7. EPS (O) and biomass (●) (A) and sugars and organic acids (B) obtained from the cultivation of *Lactobacillus confusus* TISTR 1498 at 35°C with the mod-MRS-sucrose (100 g/l)-CW medium, pH 5.5 and agitation speed 50 rpm

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