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*Full Paper*

# Influence of pH, sucrose concentration and agitation speed on exopolysaccharide production by *Lactobacillus confusus* TISTR 1498 using coconut water as a raw material substitute

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**Abstract:** Coconut water (CW) was used as a complex nitrogen source for exopolysaccharide (EPS) production by *Lactobacillus confusus* TISTR 1498 to reduce the cost of fermentation medium. EPS production was carried out in a bioreactor using (0.5×) modified MRS-sucrose-CW medium, in which three relatively expensive complex nitrogen sources (peptone, yeast extract and beef extract) were halved relative to those present in (1×) modified MRS-sucrose medium. Fermentation parameters (pH, sucrose concentration and agitation speed) were varied in the process of optimisation. Under an optimised condition (pH 5.5, sucrose concentration of 100 g/L and agitation rate of 50 rpm), the maximum EPS level of 38.2 g/L was produced at 35°C after 30 h of cultivation. This EPS concentration (38.2 g/L) from the (0.5×) medium was higher than those produced in the (1×) modified MRS-sucrose and (1×) modified MRS-sucrose-CW media (21.3 and 31.5 g/L respectively), both having the original concentration of all three complex nitrogen sources. Thus, in a bioreactor where the pH level was properly controlled, the EPS production was greatly enhanced. Sugar concentration also played an important role in the production of EPS.

**Keywords:** exopolysaccharide, *Lactobacillus confusus*, coconut water, fermentation

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## Introduction

Exopolysaccharides (EPS) are self-produced biopolymers by a number of microorganisms and are important as a natural additive in food industry. In particular, those produced by lactic acid bacteria (LAB) have gained an increased attention due to the GRAS (generally recognised as safe) status of the microbes. In the past, EPS production by LAB generally involved the optimisation of growth condition and level of generated EPS [1-11]. The EPS concentrations obtained from LAB such as *Lactobacillus* spp. were reported with the variation in productivity that could span between 0.06 to 60 g/L [6, 12-17]. To be economically feasible, the EPS production should be in the range of 10-15 g/L [13].

EPS production by *Lactobacillus confusus*, recently known as *Weissella confusa*, has been reported, which is related to characteristics and product improvement of wheat sourdough [18-19]. A research article described the structure of EPS, produced by *Lactobacillus confusus*, which was suitable as an alternative for a linear dextran produced by *Leuconostoc mesenteroides*, and also for the production of prebiotic gluco-oligosaccharides [20].

Most LAB are fastidious microorganisms requiring complex media for optimal growth and bioactivity. Besides carbon source and other supplementary nutrients for LAB cultivation, the widely used complex nitrogen sources such as expensive yeast extract, beef extract and peptone are important necessities for growth and inevitably raise the EPS production cost. It has been estimated that the yeast extract expense accounts for 30% or more of the total production cost [21-22]. Consequently, a cheaper alternative nitrogen source should be sought after in order to minimise the cost of fermentation medium.

Coconut water has been considered as worthless by-product or inexpensive raw material in Thailand and most of it is discarded to waterways. In 2001, nearly 200,000 tons of coconut water was produced in Thailand [23]. It has also been reported by the Office of Trade Policy that coconut production rose up to 1.72 million tons in 2008 [24]. Coconut water is a nutritive source of different sugars, organic acids and trace elements. It contains about 10 mg of nitrogen per litre, in comparison to 1 g of nitrogen in 10 g of yeast extract [23]. The utilisation of coconut water as a low-cost carbon source for the production of EPS by *Agrobacterium* sp. and of scleroglucan by *Sclerotium rolfsii* MTCC 2156 was reported previously [25-26].

Earlier, we reported a preliminary study on the employing of coconut water to reduce the use of complex nitrogen sources for EPS production by *L. confusus* CMU 198 in a modified MRS-sucrose medium [27]. In that study, the concentrations of three expensive medium components (peptone, yeast extract, and beef extract) in the modified MRS-sucrose-CW medium were halved, which resulted in a maximum EPS production of 11.7 g/L in unshaken Erlenmeyer flasks without pH control. In order to enhance the EPS production further, this study was thus conducted in a bioreactor in which the pH level, agitation speed and sucrose concentration were controlled and optimised.

## Materials and Methods

*Bacterial strain and inoculum preparation*

*Lactobacillus confusus* TISTR 1498 (identical to *L. confusus* CMU 198), isolated from traditional northern Thai fermented pork (Nham), was used. The strain was obtained from Thailand

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Institute of Scientific and Technological Research (TISTR) culture collection (accession number TISTR 1498). It was maintained in the MRS medium [28] plus 60% glycerol at -80°C until use. The inoculum was prepared by recovering the frozen culture stock in 10 ml of modified MRS-sucrose broth and incubating for 24 h. In the economic point of view corresponding to the temperature in Thailand and energy saving, the temperature of incubation was selected at 35°C. The optical density measured at 650 nm ($OD_{650}$) of the resulting suspension was adjusted to 0.8, which was experimentally equivalent to 2.32 x $10^8$ CFU/ml before use.

*Media and fermentation conditions*

The MRS medium was used to maintain and recover the strain from frozen state. However, in the study on the optimisation of pH, sucrose concentration and agitation speed for EPS production, the (0.5×) modified MRS-sucrose-CW medium [27] was used. The medium consisted of (g/L): peptone (Difco Laboratories, U.S.A.) 5.0; beef extract (Difco Laboratories, U.S.A.) 2.5; yeast extract (Difco Laboratories, U.S.A.) 2.5; sucrose (Mitrphol Group, Thailand) 20; $K_2HPO_4$ (Fisher Chemical, NJ) 2.0; di-ammonium hydrogen citrate (Fisher Chemical) 2.0; $CH_3COONa$. $H_2O$ (Fisher Chemical) 7.6; $MgSO_4.7H_2O$ (Fisher Chemical) 0.1; and $MnSO_4$ (Fisher Chemical) 0.4. Tween 80 (Ajax Chemical, Australia) (1 ml/L) was also added and coconut water obtained from a local market and stored in the freezer at –25°C was used as a replacement of deionised water in the medium preparation.

Under optimised conditions, other two media, viz. the (1×) modified MRS-sucrose [27] and the (1×) modified MRS-sucrose-CW, were also used. The amounts of peptone, yeast extract and beef extract in both media were double those in the (0.5×) medium. The only difference between the pair was that coconut water was used in the latter medium in stead of deionised water to dissolve the ingredients of the medium. All media were sterilised by autoclaving at 121°C for 15 min. Batch fermentations were carried out in a 5-L bioreactor (B. Braun Biostat B., Biotech International, Pennsylvania, USA) with 3 L working volume at 35°C. The bioreactor was inoculated with 10% (v/v) inoculum. Samples were taken every 6 h during 30 h of cultivation period and analysed for EPS, biomass, sucrose and lactic acid concentrations.

The pH was controlled at 5.0, 5.5, 6.0, 6.5 and 7.0 with 1M HCl or 5M NaOH as necessary. The initial sucrose concentrations were 40, 60, 80, 100 and 120 g/L while the pH and agitation speed were kept constant at 5.5 and 50 rpm respectively. The agitation speed was varied at 25, 50 and 75 rpm while the pH was controlled at 5.5 and the initial sucrose concentration kept at 100 g/L.

*Analytical methods*

The pH was measured using a pH meter (model SA 230; Orion Research, USA). Lactic acid and sucrose concentrations were determined using HPLC (Shimadzu LC-10ATvp, Shimadzu Co., Japan) under the following conditions–column: Aminex HPX-87X (300 mm×7.8 mm) incubated in column oven (model CTO-10ASvp) at 38°C; mobile phase: 5 mM $H_2SO_4$ in deionised water; flow rate: 0.75 ml/min; detectors: UV–VIS (model SPD-10Avp) at 210 nm and RI (model RID-10). The absorbance of each sample for biomass determination was quantitated at 650 nm by a spectrophotometer (Shimadzu UV-1700, Shimadzu Co., Japan) and the corresponding biomass concentration was calculated from a standard calibration curve. The EPS concentrations were

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measured as described by Duenas et al. [7]. In brief, the supernatant obtained by centrifugation (10,000 ×g for 10 min) of the fermented broth at 4°C in order to separate the cells was used for EPS determination. To inactivate EPS degrading enzymes and to precipitate proteins, the supernatant was added with 30%(v/v) trichloroacetic acid and stored for 30 min at 4°C. The crude EPS was then isolated by cool ethanol precipitation (using 3 volumes of cool ethanol). After centrifugation (3,500×g, 15 min, 4°C), the EPS pellets were dispersed in aqueous 80% ethanol and centrifuged again (three times). The final precipitates were dried to a constant weight at 55°C. All determinations were performed in triplicate.

## Results

*Effect of pH*

pH is one of the most important factors which can influence growth and production of particular products by LAB. In this study, the EPS production was investigated in the (0.5×) modified MRS-sucrose-CW medium at pH 5.0, 5.5, 6.0, 6.5 and 7.0. Fermentation was performed at a temperature of 35°C, agitation speed of 50 rpm and initial sucrose concentration of 40 g/L, which corresponded to the level present in coconut water and in the modified MRS-sucrose medium. The favourable pH level for EPS production was found to be 5.5, the EPS production being at a maximum of 12.95 g/L ($\mu$ = 0.06 L/h, $Y_{P/S}$ = 0.3 g/g and $q_P$ = 0.2 g/L/h) after 24 h incubation period (Figure 1a and Table 1). The maximum production level of lactic acid also occurred at this pH (Figure 1c) but not the biomass production (Figure 1b). All sugars were consumed after 30 h at pH 5.5, 6.0, 6.5 and 7.0 (Figure 1d).

*Effect of initial sucrose concentration*

The sugar concentration is another essential factor that influences EPS production. In this study, the effect of initial sucrose concentration was investigated at 40, 60, 80, 100 and 120 g/L by adjustment with crystalline sucrose. Fermentation was performed at 35°C, pH 5.5 and agitation speed of 50 rpm. EPS production was enhanced with the increase in initial sucrose concentration at 40-100 g/L, whereas an adverse effect on EPS production was evident at 120 g/L (Figure 2a). Maximum EPS (38.17 g/L), minimum biomass (1.5 g/L) and minimum lactic acid (19.1 g/L) were observed at the initial sucrose level of 100 g/L ($\mu$ = 0.19 L/h, $Y_{P/S}$ = 0.39 g/g and $q_P$ = 3.7 g/L/h) (Figures 2a-c and Table 1). The use of initial sugar concentrations of 60, 80 and 100 g/L resulted in complete sugar consumption within 30 h while at 120 g/L residual sugar of about 42.5 g/L was detected (Figure 2d).

*Effect of agitation speed*

Mixing is provided to keep the fermentation broth homogeneous as well as enhance mass transfer of nutrients and air. The effect of agitation speed on EPS production was investigated in the (0.5×) modified MRS-sucrose-CW medium at 25, 50 and 75 rpm. Fermentation conditions were maintained at the optimum (35°C, pH 5.5 and 100 g/L initial sucrose concentration). The highest EPS level of 38.17 g/L was produced at the speed of 50 rpm ($\mu$ = 0.19 L/h, $Y_{P/S}$ = 0.39 g/g and $q_P$ = 3.7 g/L/h) (Figure 3a and Table 1) with accompanying lowest biomass and lactic acid concentrations (Figures 3b-c). Sugar was almost depleted after 30 h of incubation period (Figure 3d).

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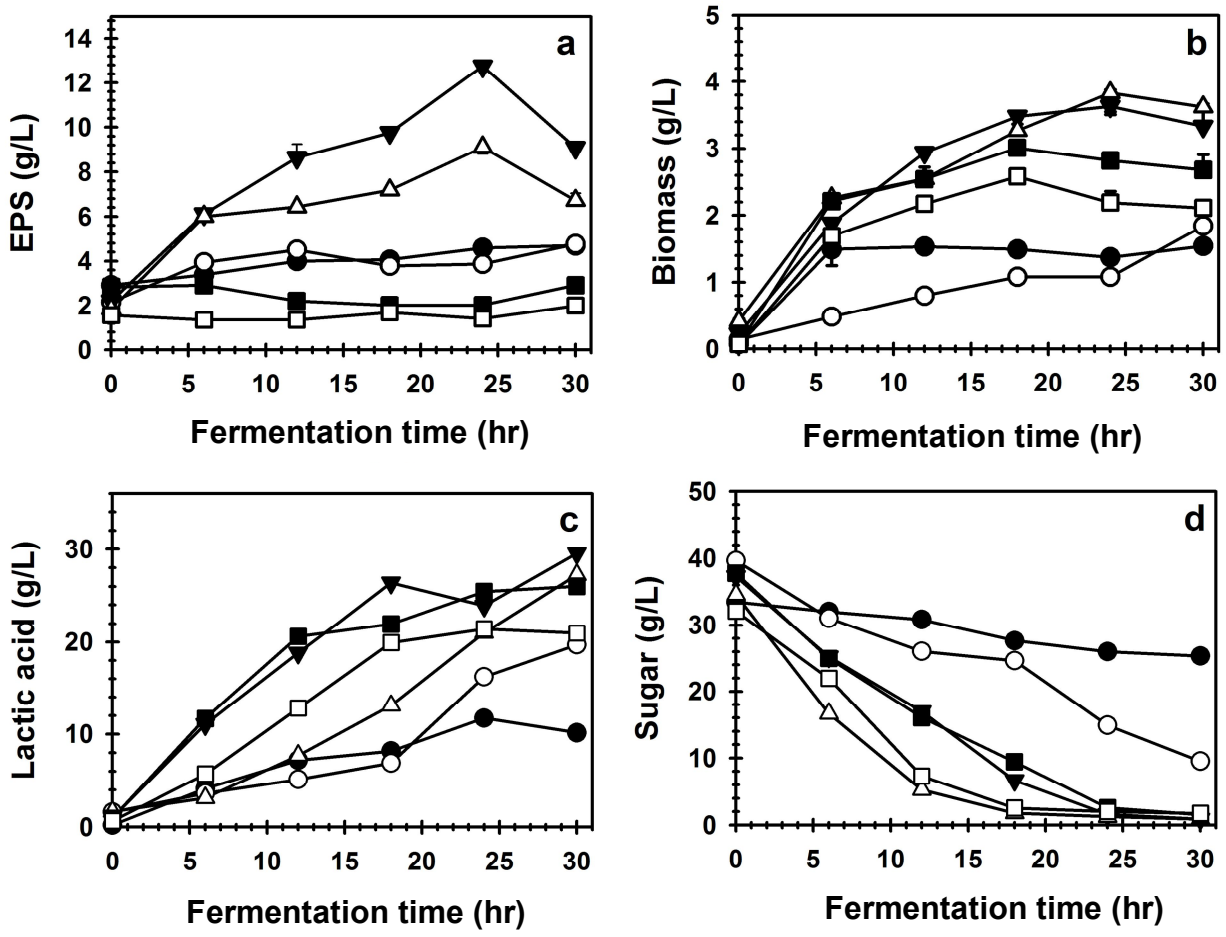


**Figure 1.** Concentrations of EPS (a), biomass (b), lactic acid (c) and sucrose (d) during the cultivation of *L. confusus* TISTR 1498 in the (0.5×) modified MRS-sucrose-CW medium (40 g/L of initial sucrose) with agitation speed at 50 rpm at different pH levels (●: uncontrolled pH fermentation, ○: pH 5, ▼: pH 5.5, △: pH 6, ■: pH 6.5 and □: pH 7)

*EPS production in the (1×) modified MRS-sucrose medium*

To investigate the effect of the medium on EPS production, a similar experimental set-up was used with (1×) modified MRS-sucrose and (1×) modified MRS-sucrose-CW as media under optimised conditions (pH 5.5, 100 g/L sucrose and 50 rpm agitation speed) at 35°C. The EPS concentrations produced were 21.35 g/L and 30.51 g/L in the two media respectively (Figure 4a).

## Discussion

This investigation focuses on the use of coconut water as a partial substitute for complex nitrogen sources, namely peptone, yeast extract and beef extract, for the production of EPS by a strain of lactic acid bacterium. It is evident that coconut water can be used to decrease the initial concentrations of the nitrogen sources in the (1×) modified MRS-sucrose medium by 50%.

The utilisation of several alternative nitrogen sources such as waste materials and agro-industrial by-products for the cultivation of lactic acid bacteria have been reported. These include malt

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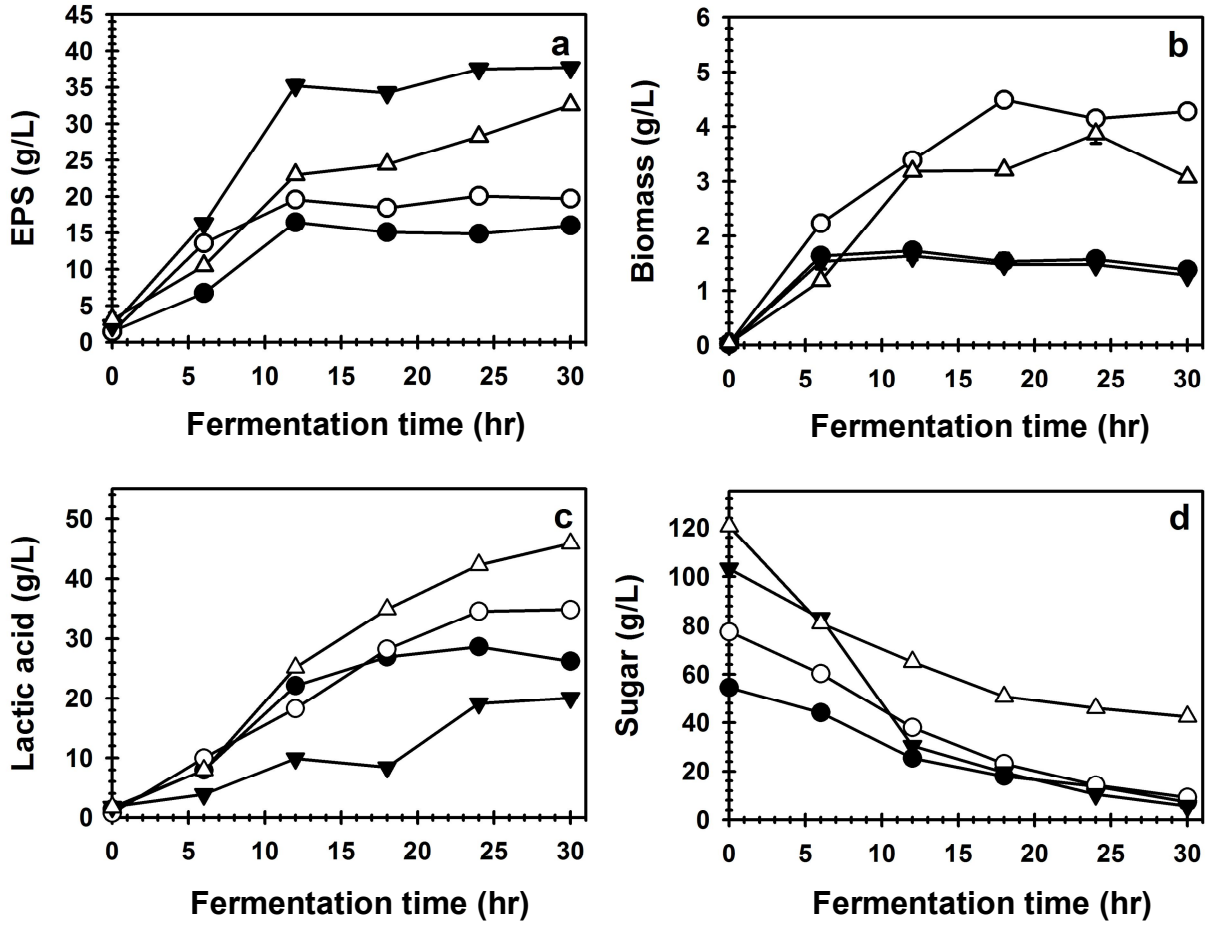


**Figure 2.** Concentrations of EPS (a), biomass (b), lactic acid (c) and sucrose (d) during the cultivation of *L. confusus* TISTR 1498 in the (0.5×) modified MRS-sucrose-CW medium, with pH = 5.5 and agitation speed = 50 rpm, at different initial sucrose concentrations (●: 60 g/L, ○: 80 g/L, ▼: 100 g/L, and △: 120 g/L)

combing nuts [21] and corn steep liquor-supplemented acid hydrolysate of soybean meal [22] for lactic acid production, as well as whey permeate [6] for exopolysaccharide production, while ram horn hydrolysate was applied to achieve both lactic acid and exopolysaccharide production [29-30].

With regard to the effect of pH on EPS production, higher EPS concentrations are usually produced under controlled pH condition [e.g. 1-2, 4-5, 10]. According to this study, the most favourable pH level for fermentation by *L. confusus* TISTR 1498 is 5.5, which resulted in the EPS yield of 12.95 g/L (at sucrose concentration of 40 g/L). The amount of EPS produced might or might not have any link with the biomass production [31-32]. From this finding, the optimal pH level of EPS production by *Lactobacillus confusus* TISTR 1498 was not found to be in the range between 6-6.7 as reported elsewhere [2-3, 8, 11, 27].

The type and concentration of sugar plays an important role in the production of EPS [8-11, 25]. In our previous study, the strain CMU 198 (identical to TISTR 1498) only produced EPS in the presence of sucrose as carbon source [27]. The optimal initial sucrose concentration of 100 g/L for the

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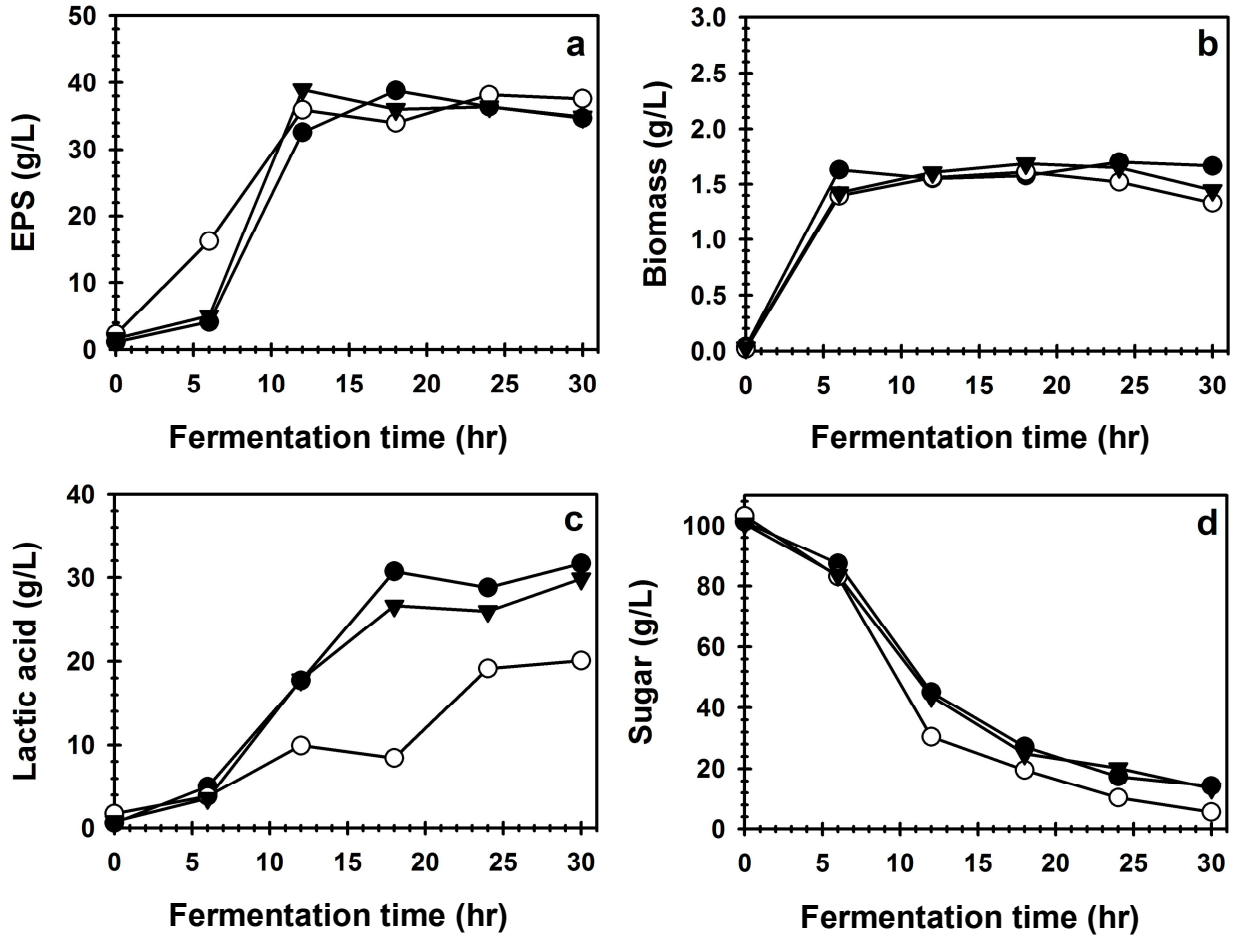


**Figure 3.** Concentrations of EPS (a), biomass (b), lactic acid (c) and sucrose (d) during the cultivation of *L. confusus* TISTR 1498 in the (0.5×) modified MRS-sucrose-CW medium (100 g/L of sucrose) with pH = 5.5 at different agitation rates (●: 25 rpm, ○: 50 rpm, and ▼: 75 rpm)

strain TISTR 1498 is at the same level as that for *Lactobacillus sp.* strain LB80 [33] and *L. reuteri* ATCC 55730 [34]. The optimal sugar concentration achieved by several lactic acid bacteria ranges between 20-100 g/L [12, 25, 33]. Higher level of sugar concentration can be detrimental to the microbial growth due to unfavourable osmotic pressure [35]

Mixing is provided in order to keep the fermentation broth homogeneous and, in some cases, aerated. In this study, the agitation rate of 50 rpm was enough to provide proper mixing. The agitation rate of 100 rpm was used for the cultivation of *Streptococcus thermophilus* LY03 [3] and *L. reuteri* ATCC 55730 [31]. Continuous agitation at 150 rpm showed a negative effect on growth and production of EPS by *Pediococcus damnosus* IOEB 8801 [9] but a maximum production of the exo-biopolymer by *Cordycep militaris* was achieved at this speed [36]. An elevation in the agitation speed also decreased EPS production by *Aureobasidium pullulans* [37] and *Enterobacter cloacae* WD7 [38].

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**Table 1.** Kinetic parameters at different pH, agitation speeds and sucrose concentrations for EPS production and growth of *Lactobacillus confusus* TISTR 1498 in (0.5×) modified MRS-sucrose-CW medium (30 h of incubation)

| Variable | EPS (g/L) | $\mu$ (L/h) | $Y_{X/S}$ (g/g) | $Y_{P/S}$ (g/g) | $q_S$ (g/L/h) | $q_P$ (g/L/h) |
|---|---|---|---|---|---|---|
| **pH**[1] | | | | | | |
| Uncontrolled | 4.69±0.29 | 0.21±0.14 | 0.18±0.060 | 0.22±0.040 | 1.17±0.037 | 0.26±0.030 |
| 5 | 4.81±0.34 | 0.04±0.003 | 0.06±0.007 | 0.09±0.002 | 0.66±0.038 | 0.06±0.002 |
| 5.5 | 12.95±1.07 | 0.06±0.002 | 0.09±0.004 | 0.30±0.011 | 0.67±0.073 | 0.20±0.017 |
| 6 | 8.81±0.82 | 0.09±0.012 | 0.10±0.005 | 0.20±0.012 | 0.90±0.051 | 0.18±0.031 |
| 6.5 | 3.10±0.21 | 0.05±0.001 | 0.10±0.030 | 0.01±0.003 | 0.50±0.008 | 0.01±0.008 |
| 7 | 1.90±0.09 | 0.03±0.002 | 0.08±0.002 | 0.01±0.001 | 0.40±0.017 | 0.01±0.005 |
| **Agitation speed (rpm)**[2] | | | | | | |
| 20 | 36.42±2.74 | 0.16±0.014 | 0.02±0.002 | 0.38±0.012 | 8.00±0.287 | 3.04±0.147 |
| 50 | 38.17±3.21 | 0.19±0.008 | 0.02±0.003 | 0.39±0.025 | 9.50±0.139 | 3.70±0.028 |
| 75 | 36.40±3.78 | 0.16±0.006 | 0.02±0.001 | 0.43±0.034 | 8.00±0.248 | 3.44±0.219 |
| **Initial sucrose concentration (g/L)**[3] | | | | | | |
| 40 | 12.95±1.47 | 0.06±0.002 | 0.09±0.008 | 0.30±0.012 | 0.67±0.041 | 0.20±0.011 |
| 60 | 16.38±1.82 | 0.09±0.001 | 0.07±0.004 | 0.29±0.019 | 1.28±0.098 | 0.37±0.026 |
| 80 | 20.55±2.98 | 0.10±0.009 | 0.06±0.003 | 0.30±0.027 | 1.67±0.053 | 0.51±0.037 |
| 100 | 38.17±3.89 | 0.19±0.018 | 0.02±0.001 | 0.39±0.032 | 9.50±0.182 | 3.70±0.042 |
| 120 | 32.65±2.41 | 0.06±0.001 | 0.04±0.002 | 0.38±0.024 | 1.50±0.002 | 0.57±0.021 |

Notes :

X = Microbial biomass; S = Substrate; P = Product

$\mu$ = Specific growth rate = $\frac{1}{X}\frac{dX}{dt}$

$Y_{X/S}$ = Yield coefficient for cells = $\Delta X/\Delta S$

$Y_{P/S}$ = Yield coefficient for EPS = $\Delta P/\Delta S$

$q_S$ = Specific substrate consumption rate = $\mu/Y_{X/S}$

$q_P$ = Specific EPS production rate = $Y_{P/S}\times q_S$

[1] Initial sucrose concentration = 40 g/L, agitation speed = 50 rpm

[2] pH = 5.5, initial sucrose concentration = 100 g/L

[3] pH = 5.5, agitation speed = 50 rpm

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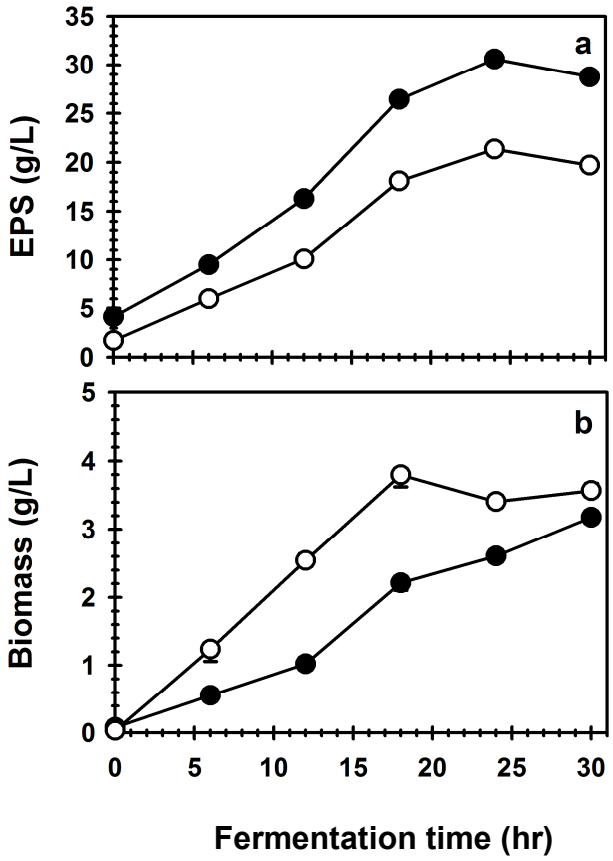


**Figure 4.** Concentrations of EPS (a) and biomass (b) during the cultivation of *L. confusus* TISTR 1498 (pH = 5.5, sucrose = 100 g/L, agitation rate = 50 rpm) in (1×) modified MRS-sucrose (○) and (1×) modified MRS-sucrose-CW (●)

The effect of pH control is strongly pronounced again here when the (1×) modified MRS-sucrose and (1×) modified MRS-sucrose-CW media gave higher levels of EPS production (Figure 4a) when compared to our previous study under uncontrolled fermentation, which gave a maximum of only 4.6 g/L of ESP in similar media [27]. However, the (0.5×) modified MRS-sucrose-CW medium used in this study was observed to further increase ESP yield. Presertsan et al. [38] suggested that a high C/N ratio may play an important role in producing a high yield of EPS with lower cell growth.

Since the concentrations of peptone, beef extract and yeast extract could be decreased by half as shown in this study, the cost of the (0.5×) cultivation medium in such a system in turn could be demonstrated to decrease from 3.47 to 1.88 THB/g EPS or nearly twofold. In Table 1, under the condition of 100 g/L initial sucrose concentration, it is seen that high EPS yield with $Y_{P/S}$ of 0.39 reflects a low biomass production with $Y_{X/S}$ of 0.02. These results are similar to those in the report of Presertsan et al [38].

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## Conclusions

Regarded as an agricultural waste that can cause pollution of the environment, coconut water, one of the naturally renewable resources, was used to partially replace complex nitrogen sources (peptone, yeast extract and beef extract) in the modified MRS-sucrose medium for EPS production by *Lactobacillus confusus* TISTR 1498. Under optimised and controlled conditions (pH 5.5, 100 g/L total sugar concentration and 50 rpm agitation speed), a high amount of ESP (about 38.2 g/L) was produced in a bioreactor. The cost of such fermentation could thus be significantly reduced.

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