การใช้ผลพลอยได้จากอุตสาหกรรมการเกษตรเป็นอาหารราคาถูกในการผลิตเอ็กโซโพลีแซคคาไรด์คีเฟอรัน
Utilization of Agro-industrial Byproducts as Low-cost Media for Production of Exopolysaccharides, Kefiran

Apisara Iadcharoen[1], Benjamas Cheirsilp[2]
Email: Benjamas.che@psu.ac.th
Biotechnology for Bioresource Utilization Laboratory, Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Songkhla 90112 Thailand

บทคัดย่อ

คีเฟอรันเป็นเอ็กโซโพลีแซ็คคาไรด์ที่สามารถใช้เป็นสารเพิ่มความคงตัวและมีคุณสมบัติเป็นสารต้านเชื้อจุลินทรีย์ อย่างไรก็ตามต้นทุนการผลิตคีเฟอรันค่อนข้างสูงเนื่องจากต้นทุนของอาหารเลี้ยงเชื้อ ดังนั้นงานวิจัยนี้จึงสนใจใช้ผลพลอยได้จากอุตสาหกรรมเพื่อเป็นแหล่งอาหารราคาถูก ซึ่งได้แก่ น้ำมะพร้าวแก่และเวย์แลคโตสในการเลี้ยงเชื้อ*L. kefiranofaciens*ผลการทดลองพบว่าน้ำมะพร้าวแก่เป็นแหล่งอาหารที่ดีต่อการผลิตคีเฟอรันซึ่งสามารถให้ผลผลิตคีเฟอรันสูงเทียบเท่ากับการใช้น้ำตาลบริสุทธิ์ และพบว่าสภาวะที่เหมาะสมในการผลิตคีเฟอรันจากน้ำมะพร้าวแก่คือ การใช้น้ำมะพร้าวแก่ที่มีความเข้มข้นน้ำตาลที่50กรัมต่อลิตร และควบคุมพีเอชที่ 5.5 ตลอดการหมักเพื่อลดการยับยั้งโดยพีเอชที่เป็นกรดและกรดแลคติก ภายใต้สภาวะที่เหมาะสมพบว่าเชื้อสามารถเจริญและให้น้ำหนักเซลล์สูงสุดเท่ากับ 3.10 กรัมต่อลิตร และมีการผลิตคีเฟอรันเท่ากับ 2.23กรัมต่อลิตร และพบว่าเชื้อมีการใช้น้ำตาลมากถึงร้อยละ 98 และมีการผลิตกรดรวมร้อยละ 5.59ผลการศึกษานี้แสดงให้เห็นว่าน้ำมะพร้าวแก่สามารถใช้เป็นแหล่งอาหารที่เหมาะสมต่อการผลิตคีเฟอรันต้นทุนต่ำ

คำสำคัญ: คีเฟอรัน, เชื้อ*Lactobacillus kefiranofaciens*, น้ำมะพร้าวแก่

ABSTRACT

Kefiran is functional exopolysaccharide that can be used asstabilizers and also has antimicrobial properties. However, the production costs of kefiran are high, mainly due to the high cost of fermentation media. Therefore, utilizing byproducts from agro-industries would be economically attractive. Mature coconut water (MCW) and whey lactose (WL) are byproducts from industries that could be as low-cost nutrient sources for kefiran production by *L. kefiranofaciens*.It was found that MCW was a good nutrient source for kefiran production as it gave high kefiran yield comparable to that obtained from pure sugars. The optimal conditions for kefiran production from MCW were: sugar concentration of 50 g/L and controlling pH at 5.5 during fermentation to reduce the inhibitory effect from acidic pH and lactic acid. The maximum dry cell weight and kefiran production were 3.10 g/L and 2.23 g/L, respectively. The sugar consumption and total acid production were also as high as 98%and 5.59%, respectively. These results indicate that MCW could be used as a suitable low-cost nutrient source for kefiran production.

Keyword: Kefiran, *Lactobacillus kefiranofaciens*, mature coconut water

INTRODUCTION

Lactic acid bacteria (LAB) that produce exopolysaccharides (EPSs) play a major role throughout the dairy industry because of their contribution to the consistency of fermented milk products, such as yoghurt, cheese, kefiretc. The EPSs can be considered as natural bio-thickeners because they are produced by LAB, which are considered as Generally Recognized As Safe. EPSs from LAB is kefiran, a type of water soluble polysaccharide that is produced by *L. kefiranofaciens* [1]. Kefiran is a repeating structure that consists of equal of glucose and galactose [2, 3]. Some researchers reported that kefiran with different chemical structure could be by changing the composition of medium [4]. The optimization of kefiran production generally proceeds via the determination of the best carbon source suitable for cell growth and kefiran production. Suitable carbon sources can be either complex or simple such as glucose, sucrose, fructose, xylose and lactose. Many studies attempted the development of an industrial medium for kefiran production. The present work focused on the development of low-cost production medium for kefiran production by*L. kefiranofaciens* JCM 6985.

Thailand produces large volumes of mature coconut water (MCW) as a waste from the coconut milk industry and most of it is discarded into the drain. The amount of mature 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 [5]. As MCW contained high amount of sugars, trace elements and nitrogen as minor components, it is considered as a good substrate for microbial cultivation [6-9]. Whey lactose (WL) is a waste from cheese production that represents a major pollution problem for countries depending on the dairy economics. As it contains high amount of lactose, it is also considered as a carbon source for microorganisms [10-12]. The utilization of MCW and WL as low-cost substrates for kefiran production could reduce the production cost of kefiran and value add the industrial wastes.

LITERATURE REVIEWS

1. Kefiran structure of kefiran consists of almost equal amounts of glucose and galactose (glucogalactan). This carbohydrate polymer was first isolated from kefir grain by have mainly produced by *L. kefir* sp. In its structure a repeating Penta saccharide unit is observed that displays in random positions one to two monosaccharide branches.

2. Influencing factors for kefiran production Carbon source: carbon sources are a very important component of cultivation medium. Since it is generally used as source for energy, microbes to growth cell and biosynthesis of kefir.[3]Studied kefiran production by mix culture form kefir grains. Using fresh milk as a culture medium and add a variety of

carbon sources at 50 g/l of fructose, glucose, sucrose and lactose. It was found that addition of carbon source to lactose resulted in the highest increase weight of kefir grain about 12 g/l and kefiran product to 4.3% about 0.53 g/L of weight's kefir grain. The results show that dry cell weight and kefiran product are the highest in case of lactose addition, which is probably due to the consumption of galactose (produced in lactose hydrolysis) required for kefiran biosynthesis.

Nitrogen source: nitrogen source is generally known for supporting cell growth and kefiran production. Nitrogen sources used in microbial cultures contain both organic nitrogen sources, such as yeast extract, triton and amino acid components, such as proteins and inorganic nitrogen sources, such as ammonium salts and nitrate salt. [13] Studied Development of cultivation medium for high yield kefiran production by *Lactobacillus Kefiranofaciens* was obtained in 50 g/l lactose supplemented culture, pH initial 5.5, 30 ℃, 72 hour by various yeast extract, peptone, extracts and casein hydrolysis. It has been found that yeast extract is a suitable nitrogen source for Kefiran production, followed by Peptone casein hydrolysis. Yeast extract produce kefiran about 0.71 g/l. In addition, the research studied the concentration of yeast extract differently on cell growth and Kefiran production by ranged from 0 to 14 g /l. The results showed that the production of Kefiran significantly at the concentration of 0-12 g / L, the highest yield of Kefiran was 1.25 g / l at 12 g/l the concentration.

pH: Cheirsilp et al [14] found that cultivating *L. kefiranofaciens* JCM 6985 in Man-Rogosa Sharpe Lactose (MRSL) medium containing pH 5.5 ranged. It's ranged sutible for kefiran product which follow Taniguchi et al. (2001) was found that the cultivation *of L. kefiranofaciens* JCM 6985 in MRSL medium pH 5.5 Kefiran product up to 460 and 650 mg/l. The results of *L. kefiranofacie* as well as Wang et al. (2008) study on the effect of pH on kefiran production by *L. kefiranofaciens* JCM 6985 with pH in the range of 3.0- 8.0 found that pH below 3.0 or more than 8.0 was not cell growth. For the production of Kefiran it was found highest kefiran production at pH 5.5 which was 0.46 g / L.

OBJECTIVES

1. To use low-cost mature coconut water and whey lactose as nutrient sources for kefiranproduction.

2. To optimized culture condition for kefiran production.

RESEARCH SCOPE

This research compared the cell growth and kefiran production of *Lactobacillus kefiranofaciens* JCM 6985 when cultivated using mature coconut water and whey lactose. The technical and economical results were compared with the use of pure sugars. The

effects of sugar concentration and pH control on cell growth and kefiran production were investigated.

MATERIALS AND METHODS

Microorganism

Microorganisms *Lactobacillus kefiranofaciens* JCM 6985, which was used to produce kefiran in this study, was obtained from Japan Collection of Microorganisms, RIKEN, Japan. The cultures were stored at -20 °C in 30% glycerol until required and were reactivated in commercial Man-Rogosa Sharpe (MRS) broth medium at 30 ±2 °C and an initial pH of 5.5for 48 h.

Medium

Modified MRS medium was composed of 20 g/L tryptone, 20 g/L meat extract, 10 g/L yeast extract, 30 g/L glucose, 1mL/L polysorbate 80, 4 g/L tri-ammonium citrate, 5g/L sodium acetate, 0.58 magnesium sulphate, 0.28 g/L manganese sulphate, 0.74 calcium chloride and with an initial pH of 5.5. The medium for kefiran production was modified MRS-whey lactose medium in which glucose was replaced by whey lactose (WL). The mature coconut water (MCW) was used directly without other nutrient supplementation. The main compositions (g/L) of the mature coconut water employed in this study were fructose, 6.4 glucose, 16.7 and sucrose 11.5 and protein 0.11.

Culture conditions

The seed culture of *L. kefiranofaciens* JCM 6985 was inoculated into 100 mL of modified MRS medium with various carbon sources including glucose, lactose and whey lactose at sugar concentration of 30 g/L and incubated at 30°C for 120 h. The cultivation was performed anaerobically and slowly stirred by a magnetic stirrer. Mature coconut water was filter-sterilized and added with glucose to adjust the initial sugar concentration up to 50, 70g/L. The pH was controlled at 5.0-5.5 by adding 10.0 M NaOH during cultivation.

Analytical methods

Cell concentration was measured based on an optical density at 660 nm (OD660). Cell dry weight was determined [14] as follows:the culture broth was centrifuged in 4 mL eppendorf at 10,000 rpm for 10 min to precipitate the cells, the cell precipitate was washed 2 times with distilled water. Extracellular kefiran in the supernatant named as broth kefiran was precipitated by the addition of the same volume of cold ethanol (-20 °C) as that of the sample and then centrifuged at 10,000 rpm, 4 °C for 10 min. The precipitate was re-dissolved in distilled water. To remove any remaining undissolved materials, the solution was centrifuged (at the same speed as above) and the clear supernatant was again precipitated in the same way. The resulting precipitate was re-dissolved in hot distilled water. Broth kefiran was then quantified calorimetrically by adding 1 mL of anthrone reagent reagentto 0.1 mL of the broth kefiran solution. The reaction mixture was incubated for 10

min at 100 °C and cooled to room temperature. The absorbance at 620 nm was measured. The concentration of broth kefiran was calculated using the standard curve of lactose. The kefiran surrounding the cells named as capsular kefiran was extracted from the cells by boiling in distilled water at 100 °C for 30 min. The mixture was centrifuged, the clear supernatant was decanted, and the amount of capsular kefiran in the supernatant was measured using the same method. The total kefiran was the sum of broth and capsular kefiran. The concentration of total sugar was calculated using the standard curve of glucose. The total acid was determined as titratable acidity

RESULTS AND DISCUSSION

Effect of various carbon sources on cell growth and kefiran production

Carbon source is considered as important component in the cultivation medium, since it is used as an energy source for cell growth and production of kefiran. The cell growth and kefiran production were determined using four types of carbon source. Fig. 1 shows effect of different carbon source on growth, kefiran production, pH, total acid (TA, %) and total sugar consumption by *L. kefiranofaciens* JCM 6985. It was found that*L. kefiranofaciens* JCM 6985 could grow on whey lactose (WL) and mature coconut water (MCW) comparable to those grown on glucose and lactose. The obtained dry cell weight were in the range of 2.2-2.7 g /L. It should be noted that MCW could be used without the addition of other nutrients possibly because MCW also contained vitamin B12, amino acids and many minerals such as phosphate that can promote the bacterial growth [16]. For kefiran production, lactose, WL and MCW gave the highest kefiran production of about 1.82-1.96 g/L. While glucose gave low kefiran of 1.29 g/L. Although lactose has been reported that it was suitable for kefiran production [16], its price is still high.

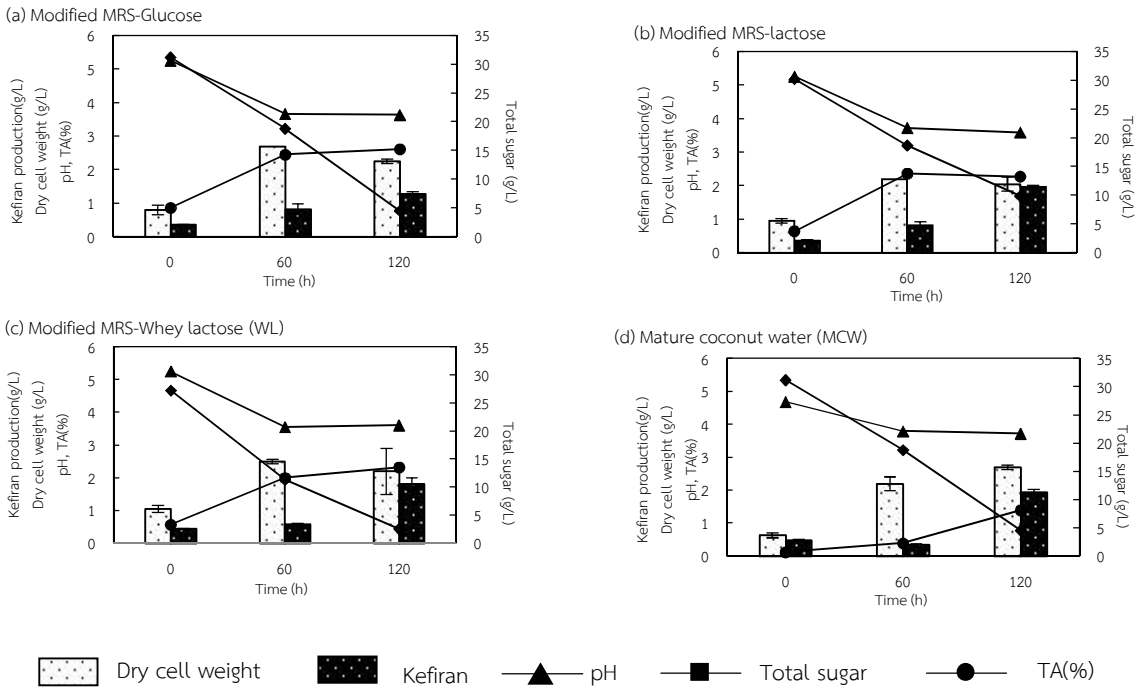

Fig. 1 Effect of different carbon source on growth, kefiran production, pH, total acid (TA, %) and total sugar consumption by *L. kefiranofaciens* JCM 6985.

Fig. 2 shows the yields of dry cell weight and kefiran. MCW gave the highest dry cell weight yield of 0.09 g-cell/g-kefiran. MCW also gave comparable kefiran yield to those of lactose. When comparing the cost of carbon source for kefiran production, MCW required the lowest cost of 1.05 Baht/g-kefiran, while the cost of modified MRS- glucose, lactose, and WL and other nutrients in MRS were as high as 81.55, 59.39 and 62.20 Baht/g-kefiran, respectively (Table 1).The cost of MRS medium was high due to the high prices of complex nitrogen sources. In the economic analyses, the cost of peptone, yeast extract and beef extract was estimated to be over 30% to the total production cost [17, 18]. Therefore, MCW was selected as the most cost-effective carbon source for kefiran production

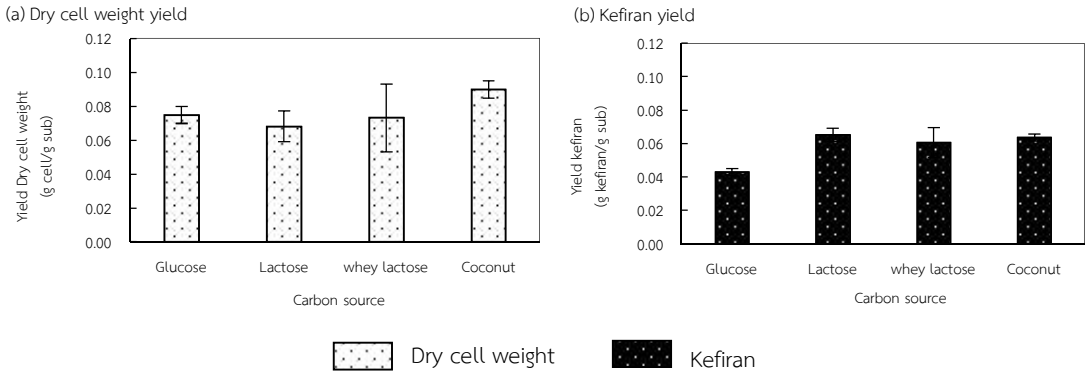

Fig. 2 Comparative effect of different carbon sources on dry cell weight yield (g/g) (a) and (b) kefiran yield (g/g) by *L. kefiranofaciens* JCM 6985. Data were taken after submerged cultivations for 120 h.

Table 1 Effect of carbon source on kefiran production and cost analysis

Carbon source (sugar 30 g/L)	Kefiran (g/L), A	Cost of carbon source (Baht/L), B	Cost of modified MRS (Baht/L), C	Cost of kefiran (Baht/g-kefiran), (B+C)/A
Modified MRS-Glucose	1.29	3.2	102	81.55
Modified MRS-Lactose	1.96	14.4	102	59.39
Modified MRS-Whey lactose	1.82	11.2	102	62.20
Coconut mature	1.91	2.0	-	1.05

Effect of initial sugar concentration and pH control on cell growth and kefiran production

To increase kefiran production from mature coconut water, glucose was added to vary the initial sugar concentration at 30-70 g/L and filter-sterilized before cultivation. The initial pH was 5.5. The results are shown in Fig. 3. The dry cell weight using three levels of initial sugar were in the same range of 2.3 to 2.7 g/L. For kefiran production, sugar concentrations of 30-50 g /L gave the highest kefiran production of about 1.91-1.99 g/L. While sugar concentration of 70 g/L gave lower kefiran of 1.45 g/L. It was found that the pH decreased rapidly in 24-48 h from pH 5.0-5.5 to 3.6-3.8, which was caused by lactic acid production. The sugar concentration of 50 g/L gave the highest total of acid 2.10 %. When using initial sugar 30 g/L, the residual total sugar was only 1.48 g/L. But when using high initial sugar of 50 g/L and 70 g/L, the residual sugars were as high as 6.69 g/L and 24.03 g/L, respectively. The lower kefiran production and lower sugar consumption could be explained

by substrate inhibition at high concentration. The kefiran production in this study was higher than those previously reported by Wang et al. [4]who used 100 g/L of glucose as source for kefiran production by*L. kefiranofaciens* JCM 6985 and found that the kefiran production was only 1.60 g/L. Kongruang [16] evaluated the effect of sucrose concentration at 20-60 g/L, but the maximum kefiran production was only 0.83 g/L.

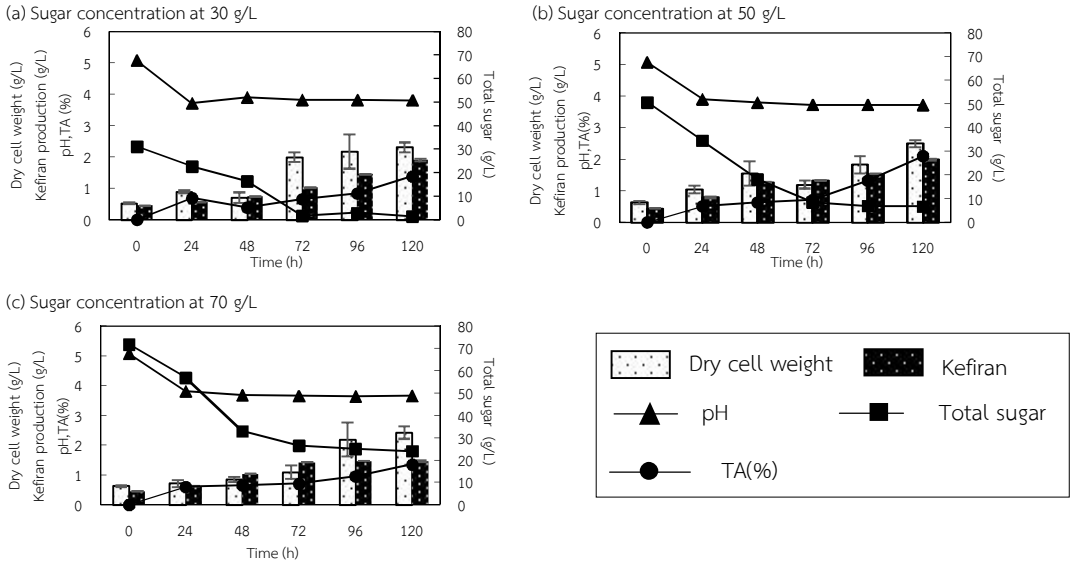

Fig. 3 Effect ofinitial sugar concentration on growth, kefiran production, pH, total acid (TA, %) and total sugar consumption by *L. kefiranofaciens* JCM 6985 under condition without pH control.

Generally, *L. kefiranofaciens* JCM 6985 produces lactic acid as growth-associated product, which decreases the culture pH. The acidic pH could inhibit cell growth and kefiran production. Therefore, the pH control during cultivation was attempted. Fig. 4 shows the effect of initial sugar concentration on growth, kefiran production, pH, total acid (TA, %) and total sugar consumption by *L. kefiranofaciens* JCM 6985 under pH control. The results showed that pH control enhanced cell growth and kefiran production. At initial sugar concentration of 50 g/L, the maximum dry cell weight obtained was 3.10 g/L and kefiran production was 2.23 g/L. The sugar consumption and total acid production were also as high as 98% and 5.59%, respectively. Cheirsilp *et al*. [20] studied the effect of pH control on kefiran production by *L. kefiranofaciens* JCM6985 using 100 g/L lactose and found that the cells grew and gave maximum dry cell weight of 5.5 g/L and kefiran production of 2.50 g/L. Suwannee [21] studied the kefiran production by *L. kefiranofaciens* JCM6985 using lactose at a concentrations of 20 g/L and pH control at 5.0-5.5 and found that the obtained dry cell weight was 3.16 g/L and kefiran production was 1.69 g/L, which was higher than those without pH control. Therefore, it could be concluded that pH control could alleviate the inhibitory effect of acidic pH and improved cell growth and production of kefiran.

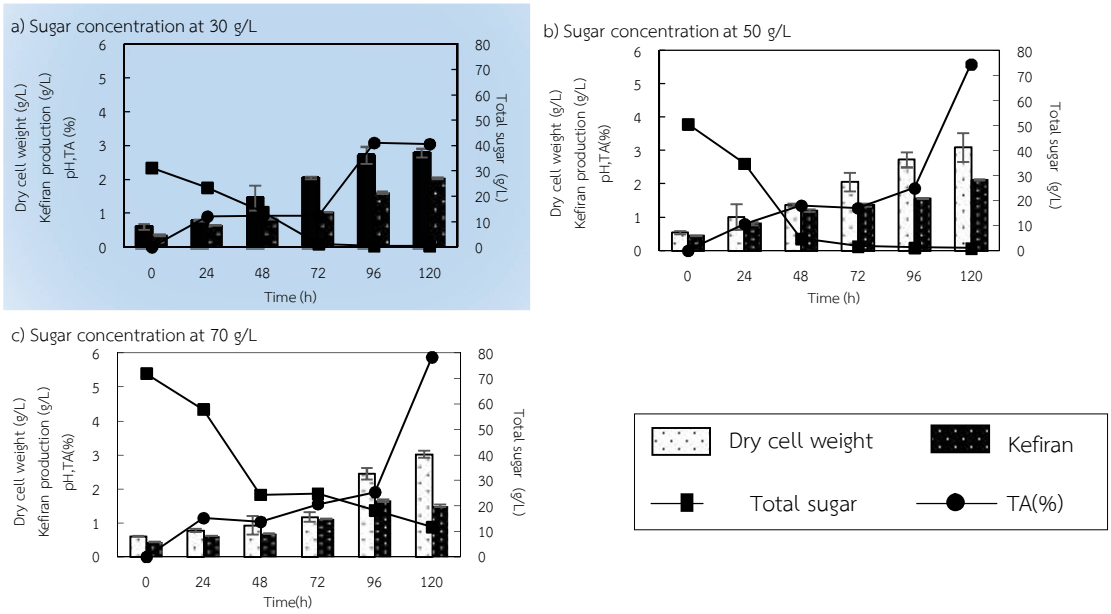

Fig. 4 Effect ofinitial sugar concentration on growth, kefiran production, pH, total acid (TA, %) and total sugar consumption by *L. kefiranofaciens* JCM 6985 under condition pH control.

CONCLUSIONS

This study demonstrated that mature coconut water (MCW), a byproduct from coconut milk industries, could be used as low-cost nutrients for kefiran production. A moderate sugar concentration of 30-50g/L in mature coconut water was suitable for both cell growth and kefiran production. The control of pH at 5.5 during fermentation could alleviate inhibitory effect from acidic pH and enhanced cell growth and kefiran production. These results indicate the promising approach to economically produce kefiran.

ACKNOWLEDGEMENTS

This research was financially supported by the Agro-Industry Practice School and Graduate School of Prince of Songkla University.

REFERENCES

[1] Kandler, O., & Kunath, P. (1983). *Lactobacillus kefir* sp. nov., a component of the microflora of kefir. Systematic and Applied Microbiology, 4(2), 286-294.

[2] Kooiman, P. (1968). The chemical structure of kefiran, the water-soluble polysaccharide of the kefir grain. Carbohydrate research, 7(2), 200-211.

[3] Zajšek, K., & Goršek, A. (2010). Mathematical modelling of ethanol production by mixed kefir grains yeast population as a function of temperature variations. Biochemical Engineering Journal, 49(1), 7-12.

[4] Wang, M., & Bi, J. (2008). Modification of characteristics of kefiran by changing the carbon source of *L. kefiranofaciens*. Journal of the Science of Food and Agriculture, 88(5), 763-769.

[5] Laws, A., Gu, Y., & Marshall, V. (2001). Biosynthesis, characterisation, and design of bacterial exopolysaccharides from lactic acid bacteria. Biotechnology advances, 19(8), 597-625.

[5] Unagul, P., Assantachai, C., Phadungruengluij, S., Suphantharika, M., Tanticharoen, M., & Verduyn, C. (2007). Coconut water as a medium additive for the production of docosahexaenoic acid (C22: 6 n3) by *Schizochytrium mangrovei* Sk-02. Bioresource technology, *98*(2), 281-287.

[7] Shivakumar, S., & Vijayendra, S. V. N. (2006). Production of exopolysaccharides by *Agrobacterium* sp. CFR-24 using coconut water–a byproduct of food industry. Letters in applied microbiology, 42(5), 477-482.

[8] Kuberski, T., Roberts, A., Linehan, B., Bryden, R. N., & Teburae, M. (1979). Coconut water as a rehydration fluid. The New Zealand medical journal, 90(641), 98-100.

[9] Survase, S. A., Saudagar, P. S., & Singhal, R. S. (2007). Use of complex media for the production of scleroglucan by *Sclerotium rolfsii* MTCC 2156. Bioresource technology, 98(7), 1509-1512.

[10] Pais, J., Farinha, I., Serafim, L. S., Prieto, M. A., Arévalo-Rodríguez, M., & Reis, M. A. M. (2009). Bioplastics production from cheese whey by recombinant *E. coli*. New Biotechnology, (25), S220.

[11] Baei, M. S., Najafpour, G. D., Lasemi, Z., Tabandeh, F., Younesi, H., Issazadeh, H., Khodabandeh, M. (2010). Optimization PHAs production from dairy industry wastewaterby *Azohydromonas lata* DSMZ 1123. Iranica Journal of Energy and Environment, 1(2), 132-136.

[12] Obruca, S., Marova, I., Melusova, S., & Ondruska, V. (2009). Production of polyester-based bioplastics by *Bacillus megaterium* grown on waste cheese whey substrate under exogenous stress. New Biotechnology, (25), S257.

[13] Dailin, D. J., Elsayed, E. A., Othman, N. Z., Malek, R. A., Ramli, S. O. L. L. E. H., Sarmidi, M. R.,& El Enshasy, H. A. (2014). Development of cultivation medium for high yield kefiran production by *L. kefiranofaciens*. In Inter. Journal of Pharmacy and Pharmaceutical Sciences. V.7, 159-163.

[14] Cheirsilp, B., Shimizu, H., & Shioya, S. (2001). Modelling and optimization of environmental conditions for kefiran production by *Lactobacillus kefiranofaciens*. Applied microbiology and biotechnology, 57(5-6), 639-646.

[15] Papanikolaou, S., Chevalot, I., Komaitis, M., Marc, I., & Aggelis, G. (2002). Single cell oil production by *Yarrowia lipolytica* growing on an industrial derivative of animal fat in batch cultures. Applied Microbiology and Biotechnology, 58(3), 308-312.

[16] Kongruang, S., & Kongtun, S. (2005, November). Quantitative analysis of exopolysaccharide production in a stirred tank bioreactor. In AIChE Annual Meeting, Conference Proceedings, Cincinnati, OH, United States, Oct.

[17] Yokoi, H., & Watanabe, T. (1992). Optimum culture conditions for production of kefiran by *Lactobacillus* sp. KPB-167B isolated from kefir grains. Journal of fermentation and bioengineering, 74(5), 327-329.

[18] Li, Z., Ding, S., Li, Z., & Tan, T. (2006). L-Lactic acid production by *Lactobacillus casei* fermentation with corn steep liquor-supplemented acid-hydrolysate of soybean meal. Biotechnology Journal: Healthcare Nutrition Technology, 1(12), 1453-1458.

[19] Venus, J. (2006). Utilization of renewables for lactic acid fermentation. Biotechnology Journal: Healthcare Nutrition Technology, 1(12), 1428-1432.

[20] Cheirsilp, B., Suksawang, S., Yeesang, J., & Boonsawang, P. (2018). Co-production of functional exopolysaccharides and lactic acid by *Lactobacillus kefiranofaciens* originated from fermented milk, kefir. Journal of food science and technology, 55(1), 331-340.

[21] Suwannee Suksawang (2016). Production of kefiran and lactic acid from Low-cost Carbon sources by *L. kefiranofaciens* JCM 6985 and the Use of Lactic Acid for Polyhydroxyalkanoate Production. Master's Thesis. Biotechnol, Department of industrial biotechnology, Faculty of agro-industry, Prince of Songkla University.