Use of Wastewater as a Substrate for Sugary Kefir Growth and Value Added Products Formation

Phattaraporn Sarikkha, Siwarutt Boonyarattanakalin, and Rachnarin Nitisoravut

***Abstract*— *Kefir is a microbial symbiotic mixture of lactic acid bacteria and yeasts. In this study, sucrose aqueous solutions and wastewaters were utilized as media for sugary kefir growth and metabolites formation. Both batch and repeated-batch conditions were explored and found the maximum kefir mass after 3 days of cultivation. For repeated-batch fermentations, the feeding cycles varied from 1-7 days and sucrose concentrations were between 1-10% (w/v). The maximum kefir mass gain was 0.47 g/100 mL in 3% (w/v) sucrose, 3- day repeated feeding cycle, and agitation rate of 90 rpm. For the maximum metabolite productions, the kefir was cultured in 10 % (w/v) sucrose solution under repeated 3-day feeding cycle for 27 days. The metabolite concentrations of lactic acid, acetic acid, and ethanol were 25.91, 5.41, and 0.71 g/L, respectively. Sugary kefir was capable of utilizing molasses and cassava in synthetic wastewaters. Molasses gave the maximum kefir mass yield of 30.95 mg/g COD removed/d. Similarly, sugar, cassava, and dairy mill wastewaters were utilized by sugary kefir. The finding offers a potential application for a preliminary treatment of food processing wastewaters as well as to convert organic matters into valuable metabolites.*

***Keywords*— Kefir grains, lactic acid, metabolites, wastewater treatment.**

1. INTRODUCTION

Kefir is a microbial symbiotic mixture of lactic acid bacteria (LAB) such as *Lactobacillus, Lactococcus, and Streptococcus* and yeasts (*Saccharomyces* spp. and *Candida* spp.) [1]-[3]. Acetic acid bacteria (AAB) may also be another important bacteria found during kefir propagation [4]. Kefir can be grown in different types of media including sugary, watery, or milky media depending on the purpose of cultivation [5]-[7]. Both bacteria and yeasts can produce biomass and metabolite products including lactic acid, acetic acid and ethanol in different proportion depending on the fermentation pathway and cultivation conditions. The effects of fermentation conditions on the milk kefir grain production, such as type and composition of the medium, substrate concentration, grain/media ratio, and fermentation time, have been investigated [8]-[11]. Additionally, environment factors such as temperature, pH, and agitation speed were also reported as being important in biomass production [6], [10]-[11]. Successful culturing conditions for kefir mass production are attained under ambient temperature, low pH and short time of cultivation [5]-[7]. Under such conditions, biomass production varied widely. Fermented products of kefir cultivation include organic acids such as lactic acid, acetic acid, propionic acid, butyric acid, and ethanol. These products are expected to add value to several industries. Lactic acid is a major fermented product which is widely used in food industries, drugs and pharmaceutical industries, cosmetics, leather and textile, and feedstocks in chemical industries.

Production of kefir mass and its value added products has gained attention in commercial production, recently. To save manufacturing costs, the use of low-cost substrates is required. Sugary kefir as defined [12] is preferred in this case because it can be cultured in minimum essential media such as sucrose solution in contrast to milk kefir which requires much more valuable substrate. The potential utilizations of sugary kefir both to biologically convert low-valued substrates to high-valued products and to treat wastewaters from food processing factories were investigated in this study. Food processing wastewaters from dairy, cassava flour, and sugar mill industries are enriched with organic matters and can be considered as potential sources of carbon. However, the fermentation conditions underlying enhanced mass production of sugary kefir were few investigated. In this study, sugary kefir was propagated in sucrose media and wastewaters to evaluate the potential for kefir mass and metabolite productions. Various conditions for kefir growth and metabolite formation were explored.

2. MATERIALS AND METHODS

Kefir grains

Sugary kefir grains were collected from the southern part of Thailand. Prior to experiments, the kefir grains were washed and inoculated in 5% (w/v) sucrose solution (brown sugar) for four cycles to eliminate influences resulting from different cultivation procedures. To further enhance kefir grain propagation, the sugary kefir grain was prepared in media by dissolving 5% (w/v)

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sucrose in mineral drinking water. After that, kefir grains were separated through a cloth-filter and then transferred to a fresh media. Kefir grain inocula were kept at 4 °C before use.

Culture media

A sucrose medium containing brown sugar at designated concentrations was used as a carbon source. It was prepared by dissolving brown sugar in dechlorinated water. The sucrose medium was also supplemented with di-ammonium hydrogen orthophosphate (DAP, 2.6% w/v) was used as nitrogen and phosphorus sources. The pH value was measured with a pH meter (METTLER TOLEDO, SevenGo™ SG2). The medium was sterilized at 121 °C for 15 min before use. The initial pH was adjusted to 7.5±0.2.

Three types of synthetic wastewater with different levels of carbohydrate complexity were 1) 5% molasses solution (w/v), 2) 1% cooked cassava starch solution (w/v), and 3) 1% raw cassava starch solution (w/v) in dechlorinated water. The synthetic wastewaters were used without sterilization in place of sucrose solution. The experiments were carried out under optimum conditions as obtained by using sucrose media. Different food processing wastewaters from sugary mill, cassava starch, and dairy mill were also investigated as low cost media for kefir growth.

Cultivation conditions

Batch fermentation was carried out in 125 mL Erlenmeyer flasks containing 100 mL of the sucrose, medium (5% w/v). Sugary kefir grains of 0.10 g in wet weight (corresponding to ~ 0.033 g in dried weight) were transferred into each flask. The experiments were carried out under room temperature (25 °C – 28 °C) without agitation (static condition) for different batch periods from 1 to 10 days.

Different repeated-batch fermentations were carried out in 5 conditions by replacing the sugar medium (5% w/v) periodically every 1, 2, 3, 5, and 7 days in each condition. For each feeding cycle, 100 mL of the sugar medium was removed and 100 mL of the fresh sugar medium was fed into each flask. The fermentation was carried out for a period of 28 days.

The effect of agitation on the kefir growth was investigated. Sugary kefir grains were cultivated under repeated-batch fermentation at room temperature for 18 days by repeated feeding of 100 mL sugar medium (5% w/v) into flasks every 3 days. The static and shaking conditions were compared. For the shaking condition, the flasks were cultivated on an orbital shaker at 90 rpm.

To optimize the substrate concentration on kefir grains production, different concentrations of sucrose media were experimented at 1, 3, 5, 7, and 10% (w/v). The fermentation was carried out under repeated-batch fermentation by shaking at 90 rpm at room temperature. The medium was fed every 3 days for 27 days.

Analyses

Kefir grains were separated from medium by filtration through filter paper (Whatman No. 1). Kefir grain mass was determined by gravimetric method. Increment of kefir grain mass was expressed as the difference between final and initial grain masses. The filtrated medium from each culture was analyzed for sugar, lactic acid, acetic acid and ethanol concentrations using high-performance liquid chromatography (HPLC, Agilent 1200 infinity Series) equipped with a VertiSep™ OA column (8 µm, 7.8 x 300 mm) at 35 °C. Sulfuric acid (0.01 N) was used as mobile phase at a flow rate of 0.8 mL/min. The aqueous samples were filtered through a 0.22 µm Millipore membrane to obtain 20 µL minimum volume of sample for analysis. The chemical oxygen demand (COD) concentration of wastewaters was determined in accordance with the procedures described in the Standard Methods [13]. Morphology of sugary kefir grains was observed with a scanning electron microscope (SEM, JSM-5410LV, JEOL Ltd., Japan), according to the modified method [12]. In brief, the grains were submerged in a phosphate buffer solution (PBS) at pH 7.2 for 24 h. The samples were then transferred to 30% glycerol. After 30 min, the sample was fractured by immersion in liquid nitrogen. The grains were post-fixed in 10 g/L osmium tetroxide dissolved in a phosphate buffer solution for 1 h at 25°C. After that, the sample was dehydrated in acetone series of 15, 30, 50 and 70%, for three times. After that, samples were critical-point dried and coated with gold using a Bal-tec SDC050 (Capovani Brothers Inc. Scotia, NY, USA).

3. RESULTS AND DISCUSSION

Kefir grain growth curve in batch conditions

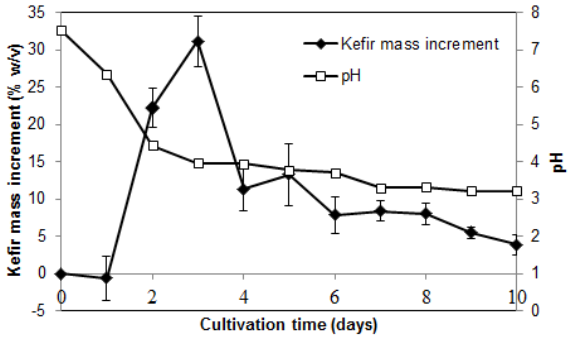

Fig.1. Profiles of percent sugary kefir mass increment and pH over a period of 10 days for batch cultivation

Fig. 1 shows kefir mass production and pH of the kefir cultures at different batch periods. In similar fashion to a typical microbial growth curve, the sugary kefir growth was observed in three stages including lag, logarithmic exponential growth, and decay phases. There was no obvious stationary phase under the experimental conditions. This is probably due to the accumulation of acid metabolites causing pH inhibition. The maximum kefir mass increment of 31.15±3.4% with kefir mass yield of 8.8 mg/g sucrose/d was obtained after 3 days of fermentation prior to die-off. Within the first 3 days, pH dropped drastically from the initial pH of 7.5±0.2 to 4.0±0.1. This level of pH was found to inhibit kefir production as reported by [10]. The optimum pH for sugary kefir was found to be at 5-5.5 [5], [10]-[11].

Rimada and Abraham [11] believed that a lower kefir mass production may also be associated with liberation of polysaccharides in the media. Ruas-Madiedo [14] reported that polysaccharide production is a bacteria self-protection mechanism in unfavorable environmental conditions such as high acidity from metabolite accumulations in the medium. The exponential growth period was found slightly varied from 1-5 days depending on experimental conditions [6]-[7], [11], [15].

Appropriate feeding cycle in repeated-batch conditions

The repeated-batch culture was carried out at different feeding cycles to enhance kefir mass production and prevent metabolite accumulations. The repeated feeding cycles were varied from every 1 to 7 days for a period of 28 days. Similar to the batch cultivation, the maximum kefir mass production in repeated-batch was also found in the 3-day feeding cycle. The maximum kefir production of 1.52 mg/g sucrose/d (0.36±0.01 g/100mL) was obtained at 12 days of 3-day repeated-batch. This is about 45.54% lower in comparison to the batch culture because the experimental conditions of the repeated-batch culture were not optimized. Cultivations with too short feeding cycles resulted in low kefir mass production. In kefir culture, some bacteria and yeast were released from kefir grains to the media and were lost during the media change. Repeated-batch cultures with short feeding cycles of 1 day could interrupt the balance of lactic acid bacteria and yeast in kefir grains. The results showed that kefir growth was increased with prolonged feeding cycles. The maximum kefir mass of 1, 2, 3, 5, and 7 days of feeding cycle were observed at 10, 12, 12, 15, and 28 days, respectively (data not shown). Repeated-batch cultures with feeding cycles longer than 3 days also produced low kefir mass production. Prolonged feeding cycles resulted in a decay phase in accordance with the results from the batch culture in which the kefir mass decreased after 3 days. Fig. 2 shows kefir mass, pH, metabolite concentrations, and metabolite yields under different feeding cycles. It can be seen that the kefir cultures with prolonged feeding cycles produced higher metabolite concentrations. The decreased metabolite yields in prolonged feeding cycles could be because of the acid stress caused by the accumulation of acidic metabolites. The repeated-batch culture with a 3-day feeding cycle yielded lactic acid, acetic acid, and ethanol in the concentrations of 12.90, 0.17, and 0.17 g/L, respectively. These levels of metabolite concentrations might not have a negative effect on kefir growth, therefore leading to the maximum kefir mass under the 3-day feeding cycle conditions. Based on the experimental results, repeated-batch fermentations with 3-day feeding cycles were carried out in further experiments to prevent metabolite inhibitions.

It is noteworthy that, although it was expected that repeated-batch propagation should be better than batch propagation due to the progressive mass gained and regular withdrawal of metabolites, it was found that less kefir mass per gram sucrose was obtained. This might be due to the disturbance of regular changes of substrate over the period of the cultivation, thereby, causing the loss of kefir mass during substrate withdrawal.

Effect of agitation conditions on kefir culture

The experiment was carried out to investigate the importance of agitation and also oxygen level under repeated-batch fermentation based on the feeding cycle of 3 days. Agitation led to a 2-fold increment of growth rate after 3 feeding cycles (9 days). Nevertheless, after 6 feeding cycles, there was only a slight difference in kefir mass between agitated and static conditions. This was a result of lowered pH due to acidic metabolites without sufficient control of pH. Thus agitation will only be beneficial if the pH is controlled and there is no accumulation of metabolites within the system. Since the focus of this study is toward practical applications of kefir for wastewater treatment, there was only a minimum control of pH. The kefir mass yield was 0.62±0.1 mg/g sucrose/d (0.34±0.1 g/100 mL). It was found that agitation is one of the important parameters for kefir growth [7], [15]-[17]. In contrast, a study by Ismaiel et al. [6] indicated that the static condition was most preferable for kefir mass production. However, in most studies, an agitation rate of 80 to 130 rpm is recommended for kefir propagation.

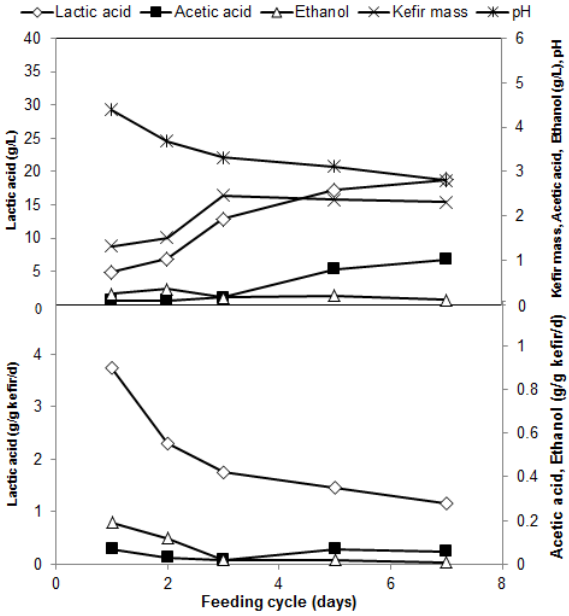

Fig.2. Profiles of metabolite concentrations, kefir mass, pH, and metabolite yields at day 28 of different feeding cycles for repeated-batch cultures

Effect of initial sucrose concentration on kefir culture

To examine the influence of substrate concentration on kefir grains production, the sugary kefir was cultured under repeated-batch conditions with a 3-day feeding cycle for a period of 27 days (9 feeding cycles) with an agitation rate of 90 rpm. The initial sucrose concentrations were varied from 1 to 10 % (w/v). In order to keep the C/N ratio constant based on the previous experiment (data not shown), the concentration of initial sucrose and the supplemented DAP (buffer salt) were fixed proportionally. Consequently, the buffering capacity of the media also increased with the increase of sucrose concentration. As a result, the ultimate pH after

27 days of kefir culture was higher with a greater sucrose concentration (see Fig. 3). The pH was varied from 3.52 to 4.12 from the initial sucrose concentrations of 1 to 10, respectively. An increase in pH should be favorable for kefir growth; however, less kefir mass was obtained with the increase in sucrose concentrations. This may be partly associated with an increase in osmotic pressure due to an increase in solute concentrations. Many previous studies found that the rate of kefir growth reduced when substrate concentration was too high [9]-[10]. Plessas and his colleagues [10] reported that less than 7.5% (w/v) of sugar in orange pulp substrate favored the kefir growth. The current study found the optimum sucrose concentration to be at 3% (w/v) sucrose. The maximum kefir mass yield was 2.11 mg/g sucrose/d (0.47 g/100 mL). The increase in buffering capacity in media with higher sucrose concentration showed a positive effect on metabolite generations. As indicated in Fig. 3, the concentrations and yields of lactic acid, acetic acid and ethanol were gradually increased with pH and sucrose concentrations. Thus, if the target of fermentation is metabolite production, a greater concentration of substrate should be provided. In regards to this aspect, the kefir propagation may be utilized for the production of metabolites by feeds from organic compounds in wastewater from food industries.

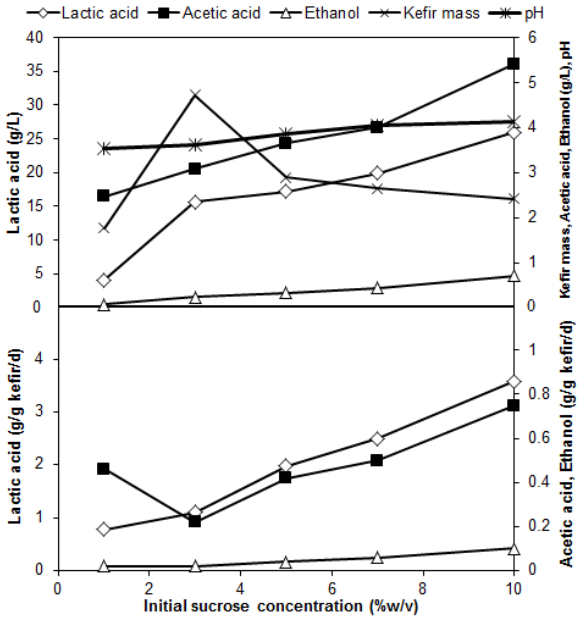

Fig.3. Profiles of metabolite concentrations, kefir mass, pH, and metabolite yields at day 27 of repeated batch with 3-day feeding cycle under different initial sucrose concentrations.

Metabolite productions

Under batch culture, metabolite formations of lactic acid, acetic acid, and ethanol were examined in association with kefir mass production and substrate utilization (see Table 1). The optimum sucrose concentration (3% w/v) with agitation rate of 90 rpm obtained from the previous section was applied in a batch culture over 10 days. The maximum kefir mass yield obtained after 3 days of batch culture was at 15.77 mg/g sucrose utilized/d which is still higher than the yield at day 3 of a batch culture with 5% (w/v) sucrose medium. Within the 8 days of culture, lactic acid was found to be the major metabolite followed by acetic acid and ethanol. As the propagation was prolonged after 8 days, acetic acid concentration was found to be higher than lactic acid. This is because acetic acid bacteria (AAB) prefer a relatively lower pH for growth than lactic acid bacteria (LAB). The maximum concentrations of lactic acid, acetic acid, and ethanol obtained were 4.13, 7.49, and 0.31 g/L, respectively, while the maximum yields were 0.128, 0.233, and 0.11 g/g kefir mass/d, respectively. These levels of concentrations were comparable to the findings of earlier studies. For kefir cultured with sucrose medium, Harta et al. [5] reported the lactic acid concentration of 8.71 g/L, while Ismaiel et al. [6] reported a very much lower lactic acid concentration of 0.43 g/L. In the same study, ethanol concentration was found to be 0.39 g/L [5]. In this study, it is speculated that a part of ethanol might also be lost due to evaporation as a result of agitation and oxidation by AAB.

Microbiological analysis

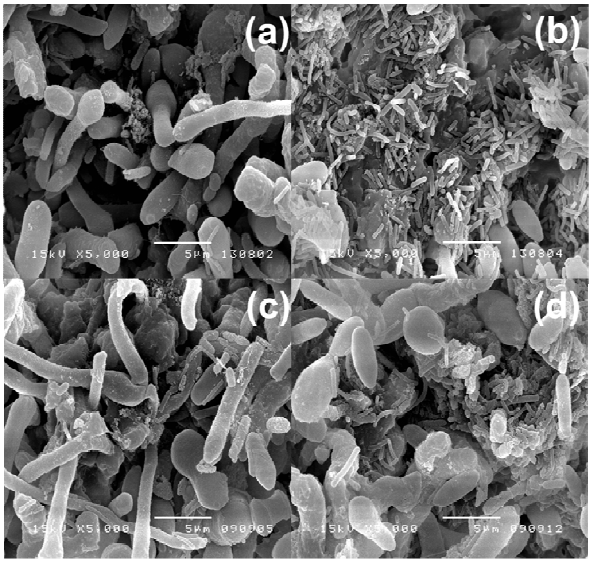

Fig.4. Scanning electron micrograph of kefir grain at outer portion (a and b) and inner portion (c and d) during day 0 and day 3 of batch culture, respectively

Fig. 4 shows the microbial community of kefir grains based on SEM examination. The samples were taken at day 0 and day 3 during batch culture. The microbial communities of sugary kefir consist of LAB, yeast as well as AAB. All of these microorganisms have the ability to convert sugar into metabolites [4]. At the beginning, the microbial population in the outer and inner sections of kefir grains was dominated by yeast cell (long and curved shape, Fig. 4(a), 4(c)). At day 3 of cultivation, the outer portion of kefir grains was mainly bacterial cells (rod shape), while the inner portion of the microbial population was mainly yeast cells (lemon shape, Fig. 4(b), 4(d)). During the initial phase of

cultivation, sucrose was hydrolyzed into glucose and fructose by the action of yeasts. Yeasts would stimulate the growth of LAB and be mainly responsible for the alcohol production. A portion of monosaccharides was converted into ethanol and carbon dioxide by yeasts. After that, LAB in kefir grains utilized monosaccharides and produced lactic acid and acetic acid which led to a lower pH value.

Wastewater as a substrate for kefir mass production and waste utilization

Most researchers focused on milk kefir propagation in various types of milk substrate [6], [15] as well as pure substrates such as glucose, fructose, and sucrose [5], [7]. Low cost waste products such as whey [9], [11], [18], discarded oranges [10], and molasses [6] were also utilized as substrates of kefir. However, the cultivation of sugary kefir in wastewater is more novel and lacks sufficient information for practical applications. In this study, sugary kefirs were cultured in different synthetic wastewaters containing high organic content. The experiment was carried out based on the results obtained in previous sections for maximum kefir mass production. Repeated-batch culture with a 3-day feeding cycle and agitation rate of 90 rpm was conducted with substrates from both synthetic wastewater and food processing wastewater. The culture was carried on for 27 days (9 feeding cycles). The kefir mass production, COD concentration and removal efficiency are reported in Table 2. for different sources of wastewater.

Molasses was found to be a favorable substrate for sugary kefir. The maximum kefir mass yield was 30.95 mg/g COD removed/d. The yields are 2.2 times higher than that obtained from sucrose solution under batch culture, which was mentioned earlier at 15.78 mg/g sucrose utilized/d or 14.05 mg/g COD removed/d (based on the unit conversion of 1.123 g COD per gram sucrose). Sugary kefir was also well propagated in other sources of wastewater with slightly lower yields. The COD removal efficiencies were widely varied within the range of 8.8 to 40.9 %, depending on the types of wastewater and concentrations. Sugary kefir was able to propagate in all types of wastewater used possibly because it contains high biodiversity of bacteria and yeasts [2], [13].

Much lower kefir mass yields were obtained in food processing wastewaters. Nevertheless, their COD removal efficiencies were found comparable to those in synthetic wastewaters. The maximum kefir mass yield was obtained at 1.91 mg/g COD removed/d for sugary mill wastewater with 35.6% COD removal. Cassava and dairy mill wastewaters were less preferable substrates for sugary kefir than sugar mill wastewater due to their more complex organic contents as can be seen from the higher COD removal in case of sugar mill.

4. CONCLUSION

Sugary kefir was well propagated in sucrose, synthetic wastewaters and food processing wastewaters. Kefir mass and metabolite productions from batch propagation showed that the 3-day feeding cycle was favorable for kefir growth while prolonged propagation was beneficial for metabolite productions. Repeated-batch propagation with a prolonged feeding cycle and high initial sucrose concentration can inhibit kefir growth as a result of higher metabolite productions. Agitation significantly boosts the kefir growth rate especially during the initial phase. The maximum kefir yield was observed under the culture conditions of 3% (w/v) sucrose, 3-day feeding cycle with agitation rate of 90 rpm, at 2.11 mg/g sucrose/d (0.47 g/100 mL). At these conditions, the concentration and yield of lactic acid, acetic acid, and ethanol were 15.62 g/L (1.10 g/g kefir mass/d), 3.08 g/L (0.22 g/g kefir mass/d), and 0.23 g /L (0.02 g/g kefir mass/d), respectively. Besides the sucrose solution, sugary kefir was able to utilize molasses and cassava starch solutions. Molasses gave the maximum kefir mass yield of 30.95 mg/g COD removed /d with 8.9% of COD removal efficiency. Similarly, food processing wastewaters can also be used as a substrate for kefir mass and metabolite productions. Sugar mill wastewater provided the maximum kefir mass yield of 1.91 mg/g COD removed /d which is equivalent to COD removal efficiency of 35.6%. Kefir cultivation presents a potential process as a preliminary treatment of wastewaters with additional benefits from valuable metabolites such as lactic acid and ethanol.

Table 1. Kefir mass and metabolite productions under batch cultures

Day	Kefir mass (g/100mL)	Residual sucrose (g/L)	pH	Metabolite concentrations (g/L)			Metabolite yields (g/g kefir mass/d)		
				Lactic acid	Acetic acid	Ethanol	Lactic acid	Acetic acid	Ethanol
0	0.1032	37.8	7.28	0.00	0.00	0.00	0.000	0.000	0.000
1	0.0998	18.7	6.19	0.00	0.00	0.00	0.000	0.000	0.000
2	0.1089	17.5	4.41	0.48	0.61	0.00	0.024	0.030	0.000
3	0.1174	13.0	3.57	1.34	0.67	0.07	0.054	0.027	0.003
4	0.1118	11.8	3.22	2.20	1.19	0.11	0.085	0.046	0.004
5	0.1086	11.5	3.08	2.33	1.55	0.22	0.089	0.059	0.008
6	0.1052	8.7	3.13	2.64	1.79	0.23	0.091	0.062	0.008
7	0.1084	8.4	3.03	3.77	2.98	0.31	0.128	0.101	0.011
8	0.1073	6.3	2.87	3.68	3.35	0.28	0.117	0.106	0.009
9	0.1045	5.6	2.85	3.85	7.49	0.20	0.120	0.233	0.006
10	0.1081	5.0	2.79	4.13	6.91	0.15	0.126	0.211	0.005

Table 2. Kefir mass production and waste utilization under different sources of carbon at 3-day repeated-batch operation

Types of Wastewater		Kefir mass yield (mg/g COD removed/d)	COD concentration		COD removal efficiency (%)
			Initial (g/L)	Final (g/L)	
Synthetic wastewater	Molasses	30.95	37.37	34.02	8.9
	Cooked cassava	12.24	20.21	14.16	29.9
	Raw cassava	9.48	18.13	10.71	40.9
Food processing wastewater	Sugar mill	1.91	3.06	1.97	35.6
	Cassava mill	0.58	29.21	25.36	13.2
	Dairy mill	1.56	4.70	3.9	17.0

ACKNOWLEDGMENT

Phattaraporn Sarikkha is a recipient of graduate scholarships of Sirindhorn International Institute of Technology, Thammasat University (SIIT-TU) and Bangchak Low Carbon Technology.

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