Potential Use of *Lactobacillus casei* TISTR 1500 for the Bioconversion of Palmyra Sap to Lactic acid

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Abstract: Production of lactic acid from palmyra sap by fermentation has been studied using *Lactobacillus casei* TISTR 1500 as the producer organism. The effects of the physical and chemical properties of palmyra sap were determined. The pH, water content, total soluble solids, total sugars, and total nitrogen were 4.3, 86.2%, 14.8°Brix, 134.0 g L^{-1}, and 0.03 g L^{-1}, respectively. The fermentation took place in a flask under static conditions. The variation of 10.0-134.0 g L^{-1} total sugars in the palmyra sap was carried out at 37°C for 24 h in a basal medium. The final lactic acid concentration, dry cell weight and productivity increased with the increment of total sugars of palmyra sap concentrations up to 134.0 g L^{-1}. The kinetic parameters of the palmyra sap of 134.0 g L^{-1} total sugars revealed that the specific growth rate (μ) was 0.05 h^{-1}, the maximum productivity (R_M) was 2.02 g lactic acid L^{-1} h^{-1}, the cellular yield coefficient ($Y_{X/S}$) was 0.20 g cell g^{-1} sugar and the lactic acid yield ($Y_{P/S}$) was 0.78 g g^{-1}.

Keyword: Palmyra sap; Lactic acid; *Lactobacillus casei* TISTR 1500; Fermentation.

1. Introduction

Lactic acid is widely used as an acidulant, flavor, and preservative in food, and in the pharmaceutical, leather, and textile industries. It is also polymerized to biodegradable polylactic acid (PLA), which is used for medical applications such as sutures and clips for wound closure or prosthetic devices. Additionally, it is used for the production of basic chemicals [1].

Lactobacillus casei strains are lactic acid bacteria with remarkable phenotypic and genotypic variability [2] that colonize diverse ecological niches, among them, the human gastrointestinal tract [3], and have broad commercial applications. The homofermentative *L. casei* is known to be an L(+)-lactic acid producer. Furthermore, *L. casei* is acidotolerant with an optimum pH of 5.5 and is relatively insensitive to product inhibition by lactic acid [4]. *L. casei* is also an anaerobic microorganism. Consequently, the microorganism grows better in a static culture where the fermentation conditions are anaerobic [5].

Manufacturing costs of lactic acid are greatly influenced by the cost of the raw materials, especially the pure ingredients such as glucose, sucrose and lactose [6]. Hence, using cheap raw materials as a fermentation substrate for lactic acid is an alternative to reduce the cost of lactic acid production. Southern Thailand has renewable and abundant agricultural resources which are mainly composed of sugar such as palmyra palms. Most plantation areas of palmyra palms are in Songkhla province with approximately 3 million plants. The most important product of palmyra palms is the sap and juice which is rich in sugars (10-17%, w/v) [7].

The aim of this study is to evaluate the possibility of using palmyra sap as a substrate for lactic acid bacterial strains to produce lactic acid.

2. Experimental

2.1 Microorganisms

L. casei TISTR 1500, the only strain used in this study, was obtained from the Department of Biotechnology, Faculty of Agro-Industry, Rajamangala University of Technology Srivijaya. The strain was provided by the Thailand Institute of Scientific and Technological Research (TISTR) and was preserved in de Man, Rogosa, Sharpe (MRS) media with the following composition (in g L^{-1}): proteose peptone, 10; beef extract, 10; yeast extract, 5; glucose, 20; polysorbate 80, 1; ammonium citrate, 2; sodium acetate 5; magnesium sulphate, 0.1; maganese sulphate, 0.05; dipotassium phosphate, 2; and agar, 15, [8] containing 20% vv^{-1} glycerol at -20°C. The culture was propagated twice in MRS medium (initial pH 5.5, 18-24 h, 37°C) prior to use as an inoculum.

2.2 Culture preparation

Ten percent of inoculum (vv^{-1}) was propagated in MRS broth or palmyra sap, which was obtained from the same source in Songkhla province, Thailand. The cultures were cultivated at 37°C for 24 h.

2.3 Physical and chemical properties measurement

We characterized three palm sap samples collected from the same source in Songkhla province, in southern Thailand. Collected sap was kept in an icebox (4°C) to inhibit the activity of microorganisms during transportation (30 min) to the department of chemical engineering, Prince of Songkla University, Hat Yai Campus.

The pH was measured at ambient temperature with a pH meter (Satorious, USA). The total soluble solids of palmyra sap were determined in degree Brix using a hand-held refractometer (RT-30 ATC, Instrutherm, Brazil). Total sugars were quantified by the phenol sulfuric method [9]. The type and concentration of sugar was determined using HPLC (System controller: SCL-10A *VP*, Liquid chromatograph: LC-10AD *VP*, Degasser: DGU-12A, RI detector: RID-10A, Auto injector: SIL-10AD *VP*, Column oven: CTO-10AS *VP*, Shimadzu, Japan) with Shim pack CLC NH_2 column. The mobile phase used was a solution of acetonitrile and water (75:25), pumped at a flow rate of 1.5 mL min^{-1} and an injection volume 10 μL. Samples were prepared by making appropriate dilutions with distilled water. All sample solutions were passed through a 0.45 μm syringe filter to remove particulates prior to HPLC analysis. The sugars of D-glucose, D-fructose and sucrose were used as external standards. The calibration curve of each sugar was plotted between peak areas and concentrations. Water content was analyzed by drying at 105°C for 24 h.

2.4 Incubation period on lactic acid production in static-flask cultivation

The starter culture (10%, vv^{-1}) was transferred into the MRS medium (90 mL) in a static incubator and the medium was adjusted to pH 5.5 with 1 N NaOH. Uncontrolled pH fermentations were performed in a final working volume of 100 mL in 250-mL Erlnemeyer flask, and cultivated at 37°C for 24 h. Samples were taken every 2 h.

2.5 Lactic acid production in batch fermentation using palmyra sap as a substrate

The 10% vv^{-1} inoculum of this culture was then transferred to a 250-mL Erlnemeyer flask containing 90 mL MRS broth (containing no glucose) with various total sugars of palmyra sap (10.0-134.0 g L^{-1}), and incubated for 24 h at 37°C in a static incubator with the medium adjusted to pH 5.5 with 1 N NaOH. Samples were withdrawn aseptically from the fermentation flasks every 4 h. The experiments were performed in triplicate.

2.6 Analytical methods

Cell growth was measured by diluting the culture broth with distilled water to obtain optimum dilution. After mixing, the absorbance was measured using a UV-spectrophotometer (UV-1601, Shimadzu, Japan) at 660 nm [10]. Dry cell weight was determined by centrifugation of the culture broth (2 mL) at 8,000 rpm for 15 min. The cell sediments were dried for 24 h at 105°C in a hot air oven (ULE, MEMMERT GMBH), and then weighed to constant weight after cooling in a desiccator [11]. Lactic acid and acetic acid concentrations in [the] supernatent were conducted by means of GC analysis. The gas chromatograph (GC-14A, Shimadzu, Japan) was equipped with BP-20 GC column (30m x 0.53mm) using flame ionization detector [12]. Residual sugar (sucrose, glucose and fructose) in the supernatent was determined by HPLC analysis, adapted from the method described by Liu [13]. Total sugars concentrations were analyzed by the phenol sulfuric acid method using sucrose as a standard [9]. Total nitrogen content was determined according to literature [14]. The concentration of minerals was determined with inductively coupled plasma (Perkin Elmer Optima 4300 DV) adapted from Kosugi [24].

The following kinetic parameters were determined: the specific growth rate (μ, h^{-1}), defined as the ratio of logarithm of biomass concentration produced to elapsed time (h); cellular yield coefficient ($Y_{X/S}$, g g^{-1}), defined as the ratio of the total cell mass present in the medium to sugar consumed; conversion yield of substrate to product ($Y_{P/S}$, g g^{-1}), defined as the ratio of lactic acid produced to sugar consumed; and maximum productivity (R_M, g L^{-1} h^{-1}), calculated as the ratio of lactic acid concentration to the fermentation time [15].

3. Results and Discussion

The physical and chemical properties of each sample was determined within a day of collection. The results showed that the pH value was 4.3. Normally, natural fresh palmyra sap has a neutral pH of approximately 7 [16-18]. Hence, a low pH indicates that the initial fermentation step of palmyra sap has occurred, for example, during collecting and transportation. Total soluble solids were 14.8°Brix. The variation of total soluble solids of palmyra sap depends on its different sources and fermentation of sugar caused by microorganisms [18]. Total sugars were 134.0 g L^{-1} and sucrose was found to be the dominant sugar in palmyra sap. Water content was 86.2%. Total nitrogen content was 0.03 g L^{-1}, and mineral content of Na, K and Mg was 0.050, 0.018 and 0.054 g L^{-1}, respectively. The overall properties measured were similar to those obtained from the literature [19-20]. Palmyra sap has a high sugar content, which was used as a carbon source for lactic acid bacterial strains to produce lactic acid. However, MRS medium, which contains yeast extract, peptone and meat extract, was supplemented with palmyra sap to support growth as the sap did not contain a sufficient amount of nitrogen [1].

Incubation period was determined on bacterial growth and lactic acid production by *L. casei* TISTR 1500 in MRS medium (pH 5.5) using 20 g L^{-1} glucose as a carbon source for 24 h at 37°C. The profile of growth (DCW), lactic acid production and total sugars utilization is shown in Figure 1. The bacteria growth [rate] rapidly reached the maximum within the first 14 h, which was correlated with the rapid decline of total sugars of palmyra sap, which in turn was due to the sugar being metabolized by cells and the cells forming lactic acid. The maximum amount of lactic acid (22.06 g L^{-1}) was produced from 20 g L^{-1} glucose within 14 h of fermentation, with an increase in dry cell weight from 1.52 to 5.05 g L^{-1}. The kinetic parameters were as follows; specific growth rate (μ) was 0.06 h^{-1}, the product yield ($Y_{P/S}$) was 1.20 g lactic acid g $sugar^{-1}$, the cellular yield coefficient ($Y_{X/S}$) was 0.20 g cell g^{-1} sugar, and the maximum productivity (R_M) was 1.58 g lactic acid L^{-1} h^{-1}.

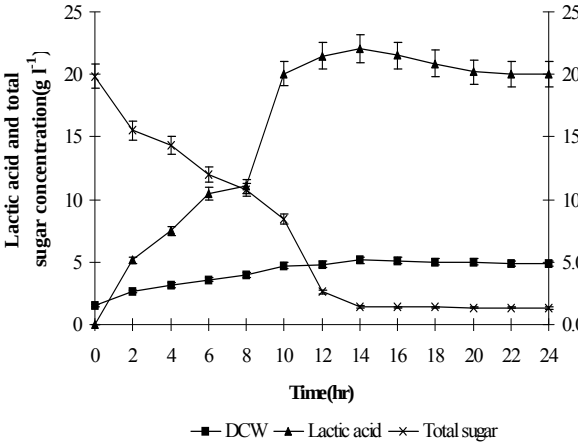

Figure 1. Incubation period on lactic acid production in static flask cultivation with MRS medium.

The value of lactic acid yield was more than one. It may be explained by the utilization of nutrient sources other than the considered sugars or by hydrolysis of the oligosaccharides, which alters the mass of sugars by the incorporation of water molecules [21].

Lactic acid fermentation from different carbohydrates was performed in a fermentation medium with carbohydrate concentration 20 g L^{-1} (Table 1). When the medium containing glucose was used as a carbon source, *L. casei* TISTR 1500 could produce lactic acid more efficiently than [when] fructose [or] sucrose [were used].

It is well known that sucrose is poorly metabolized by microorganisms compared to glucose. However, *L. casei* TISTR 1500 was able to utilize all sugars for biomass generation and lactic acid production. Our work aim is to use palmyra sap as a substrate for lactic acid production; the subsequent experiments were carried out using available palmyra sap.

Table 1. Effect of different sugars on cell growth and lactic acid production by *L. casei* TISTR 1500.

Carbon source	Initial concentrations of substrate (g L^{-1})	Dry Cell Weight (g L^{-1})	Final lactic acid (g L^{-1})
Glucose	21.65	5.22	22.06
Fructose	21.96	5.05	21.15
Sucrose	22.45	4.78	20.41

Table 2. Effect of total sugars of palmyra sap on lactic acid production dry cell weight and productivity.

Total sugars of palmyra sap (g L^{-1})	Substrate conversion[a] (%)	Final lactic acid (g L^{-1})	Dry cell weight (g L^{-1})	Lactic acid yield (g g^{-1})	Productivity (g L^{-1} h^{-1})
10.0	94.96	7.78	1.30	0.71	0.56
20.0	85.81	12.00	1.53	0.72	0.86
40.0	58.39	17.00	2.75	0.74	1.21
60.0	41.59	18.95	4.60	0.76	1.35
134.0	27.19	28.35	8.51	0.78	2.02

[a] g-sugar consumed/g-initial sugar x 100

In order to determine the influence of palmyra sap concentrations on lactic acid fermentation, *L. casei* TISTR 1500 was cultured on a static flask at 37°C, pH 5.5 for 14 h using 10.0-134.0 g L^{-1} total sugars of palmyra sap.

The results obtained (Table 2) show that final lactic acid, dry cell weight and productivity increased with the increase in total sugars of palmyra sap from 10.0 up to 134.0 g L^{-1}. This indicated that lactic acid production was not limited by substrate inhibition. Lactic acid yields based on total sugars consumed were 0.71-0.78 g g^{-1}, and the highest yield obtained was 0.78 g g^{-1} at 134.0 g L^{-1} total sugars of palmyra sap. While the maximum dry cell weight obtained was 8.51 g L^{-1} at 134.0 g L^{-1} total sugars of palmyra sap, the highest productivity of lactic acid was found to be 2.02 g L^{-1} h^{-1} at 134.0 g L^{-1} total sugars of palmyra sap.

The optimum of total sugars of palmyra sap for lactic acid fermentation by batch culture of *L. casei* TISTR 1500 seemed to be 134.0 g L^{-1} based on economical considerations of final lactic acid [amounts] and productivity.

However, not all the sugars of palmyra sap were fully consumed in the presence of high total sugars of palmyra sap and approximately 73% of total sugars of palmyra sap remained unused in the fermentation medium. The high concentrations of lactic acid and acetic acid had inhibited the use of the substrates.

The kinetic parameters of the palmyra sap of 134.0 g L^{-1} total sugars revealed that specific growth rate (μ) was 0.05 h^{-1}, the maximum productivity (R_M) was 2.02 g lactic acid L^{-1} h^{-1} and lactic acid yield ($Y_{P/S}$) was 0.78 g g^{-1}.

The profile of growth (cell dry weight), pH, lactic acid production and total sugars utilization is shown in Figure 2. The total sugars of palmyra sap concentrations of 10.0, 20.0, 40.0, 60.0, and 134.0 g L^{-1} gave rapid growth rates (maximum dry cell weight: 1.30, 1.53, 2.75. 4.60 and 8.51 g L^{-1}, respectively).

Lactic acid from *L. casei* TISTR 1500 increased as the total sugars concentrations increased up to 134.0 g L^{-1}. Therefore, the carbon source concentration affected the efficiency of substrate conversion to lactic acid. The high concentration of carbon source increased with high lactic acid concentration.

In our study, not only lactic acid but also acetic acid is one of the by-products in the fermentation broth (0-1.43 g L^{-1}). Ethanol and formic acid were not detected. Lactic acid bacteria ferment sugars via different pathways resulting in homo-, hetero-, or mixed acid fermentation. Homo-fermentation gives only lactic acid as the end product of sugar metabolism, and the Embden-Meyerhof-Parnas pathway is used. In hetero-fermentation equimolar amounts of lactic acid, carbon dioxide and ethanol or acetate are formed from sugar via the phosphoketolase pathway. This pathway is used by facultative hetero-fermenters, such as *L. casei*, for the fermentation of sugar [1].

The changes in pH during the cultivation of *L. casei* TISTR 1500 in various total sugars concentrations were similar. The pH decreased from 5.64 to 3.45 at the end of cultivation (14 h).

L. casei is an anaerobic microorganism. Consequently, the microorganism grows better in a static culture where the fermentation conditions are anaerobic. Panesar et al. [22] used *L. casei* for L(+)-lactic acid production. They found that no difference was observed for lactic acid production with agitation of fermentation broth. Moreover, Gandhi et al. [23] used stationary conditions for the lactic acid production using different lactobacilli cultures (*L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. casei* etc.).

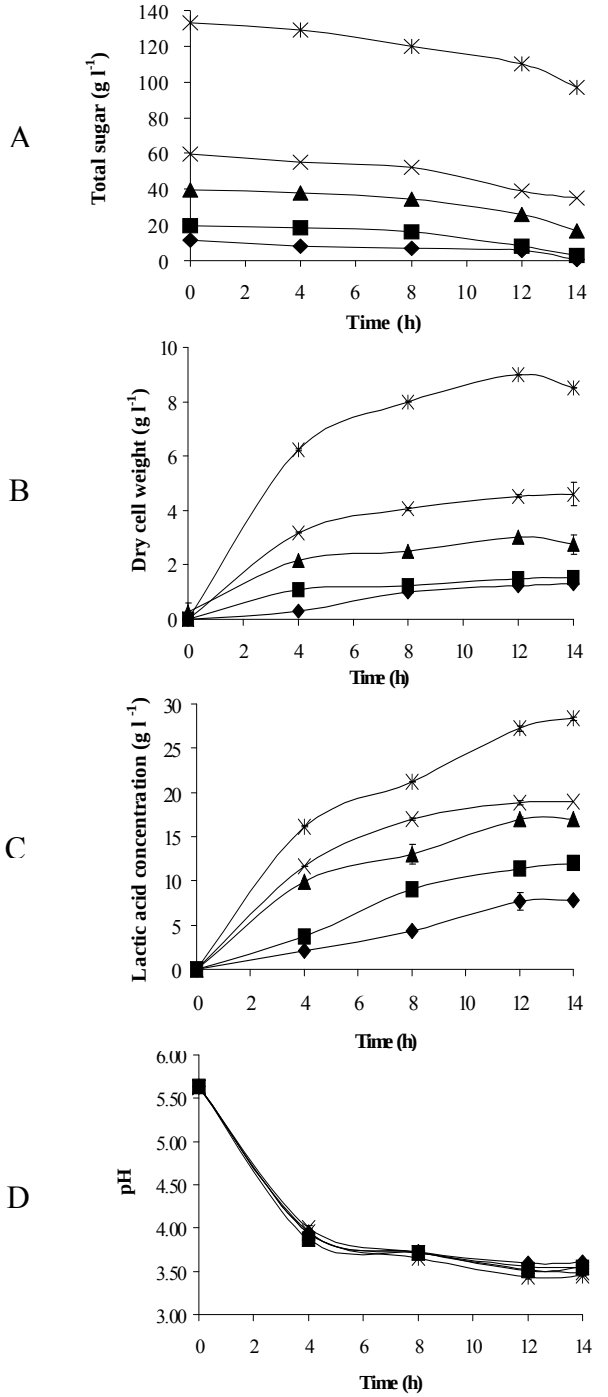

Figure 2. Kinetic profiles of total sugars consumption (A), Dry cell weight (B), Lactic acid production (C) and pH (D) over time of fermentation for lactic acid production by *L. casei* TISTR 1500 with (♦): 10 g L^{-1}, (∎): 20 g L^{-1}, (▲): 40 g L^{-1}, (x): 60 g L^{-1}, (∗): 134 g L^{-1} total sugars of palmyra sap.

Table 3. Data reported on batch fermentations for lactic acid from agricultural resources.

Microorganism	Raw material	Initial sugar (g L^{-1})	Lactic acid (g L^{-1})	Productivity (g L^{-1} h^{-1})	Reference
Lactobacillus lactis ATCC19435	Oil palm sap	18.95 (total sugars)	17.04	0.24	[24]
Lactobacillus lactis IO-1	Sugarcane bagasse	30.00 (xylose)	10.85	0.17	[25]
Lactobacillus bulgaricus	Whey	50.00 (lactose)	20.80	0.30	[26]
Lactobacillus delbrueckii	Brewer's spent grain	50.00 (glucose)	35.54	0.59	[27]
Lactobacillus delbrueckii	Hydrolyzed cane sugar	150.00 (sucrose)	128.50	3.20	[28]
Lactobacillus rhamnosus	Wheat bran	25.00 (wheat bran hydrolysate)	75.00	3.75	[29]
Lactobacillus delbrueckii NCIMB 8130	Molasses	100.00 (molasses sugar)	90.00	3.80	[30]
Lactobacillus delbrueckii Uc-3	Molasses	148.00 (molasses sugar)	129.00	4.30	[31]
Lactobacillus delbrueckii NCIMB 8130	Molasses	190.00 (molasses sugar)	166.00	4.15	[31]
Lactobacillus casei B-442	Cashew apple juice	50 (reducing sugar)	47.37	2.36	[32]
Lactobacillus casei TISTR 1500	Palmyra sap	134.00 (total sugars)	28.35	2.02	This study

With increasing interests in producing biotechnological products from low-cost and renewable biomass, production of lactic acid from various raw agricultural materials has gained considerable attention recently. Many microorganisms, [including] lactic acid bacteria (LAB), have been investigated for the production of lactic acid. Some examples of microbial lactic acid production from agricultural resources by LAB are shown in Table 3. Relatively low lactic acid concentrations were obtained when oil palm sap [24], sugarcane bagasse [25], whey [26] and brewer's spent grain [27] were used for lactic acid production. However, higher concentrations of lactic acid were reported when using hydrolyzed cane sugar [28], wheat bran [29], molasses [30-31], and cashew apple juice [32]. From the results of lactic acid concentration and productivity, palmyra sap has potential for lactic acid fermentation using *L. casei* TISTR 1500.

4. Conclusion

This study demonstrated that palmyra sap had low pH, total soluble solid and total nitrogen, but high content of total sugars mostly in the form of sucrose. It has a high potential to produce lactic acid by fermentation using *Lactobacillus casei* TISTR 1500. The maximum productivity from this bacterium strain during batch cultivation obtained 2.02 g of lactic acid L^{-1} h^{-1} with 134.0 g L^{-1} total sugars of palmyra sap at 37°C and a retention time of 14 h.

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