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Using Date Palm (Phoenix dactylifera L.) by-products to Cultivate Lactobacillus reuteri spp.

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Abstract

Lactic acid bacteria (LAB) are used by industry to produce fermented food products. The standard media used to cultivate LABs is DeMan Rogosa Sharp (MRS). However, it is expensive. Alternative low-cost media must be developed for industrial use. A good source for growth media components are by-products generated during the production of agricultural goods, such as dates. Our objective was to investigate the use of date by-products for cultivating *Lactobacillus reuteri*.

Date palm extract (DPE) was prepared by pressing fresh date fruits for one week and diluting 1:2 with diH_2O, centrifuging at 4696 x *g* and 4 ℃ 25 min, and autoclaving the supernatant at 110 ℃ for 15 min. An MRS-based buffer solution was added to DPE make a date palm medium (DPM). DPM was then enriched with various amounts phytone peptone (0, 0.2, 0.4, 0.6, and 0.8 %, w/v). The enriched DPMs were used to cultivate three strains of *Lactobacillus reuteri*: DSM 20016, CF2-7F, and SD 2112. Our results showed that in the DPM minus phytone peptone, bacterial counts reached 3.18 ± 0.5 log CFU/mL. Addition of lower amounts of phytone peptone did not improve bacterial growth. However, DPM medium supplemented with 0.8% phytone peptone improved the bacterial counts, which reached 6.94 ± 0.1 log CFU/mL, similar to what was observed with MRS (7.90 ± 0.24 log CFU/mL). There was no significant difference in the growth of LAB in MRS and phytone peptone enriched media DPM ($p > 0.05$). Date by-products are potentially alternative low cost components of LAB growth media.

Keywords: bacterial population, cultivation, date palm extract, growth medium, lactic acid bacteria, phytone peptone

1. Introduction

Lactic acid bacteria (LAB) are widely used in the fermentation of many food products such as meat, vegetables, beverages, dairy products. High cell mass production has become critical because many companies have developed an interest in LAB, not only as a starter culture, but also as a valuable probiotic additive to their food products (Barakat, Ibrahim, Tawfik, El-Kholy, & El-Rab, 2011).

Lactobacilli are fastidious microorganisms that have numerous nutritional requirements for growth, including a need for nitrogen sources (Hayek, Shahbazi, Awaisheh, Shah, & Ibrahim, 2013). However, nitrogen sources are expensive and increase the cost of the growth media. The standard medium used to grow LAB is DeMan Rogosa Sharp (MRS). MRS is expensive and the industry needs a low-cost alternative. Food waste and agricultural by-products could be used as components of a low-cost media (Cotter & Hill, 2003). Large amount of by-products are generated during the processing of a variety of agricultural products. This waste includes fruit

pomace, seeds, peels, pulps, unused flesh, and husks. These are promising sources of media components that are functional on many levels (Gao, Ma, & Xu, 2011).

The Nitrogen sources in the media are especially expensive. Many attempts have been made to use agricultural by-products to substitute the standard sources of nitrogen at a lower cost. Good examples are the use of hydrolysates of wheat bran mixed with corn steep liquor (Li, Han, Ji, Wang, & Tan, 2010), corn steep liquor supplemented with Tween 80, and K_2HPO_4 (Coelho, Augusto, & Lages, 2011), cane molasses mixed with animal and marine by-products (Khannous et al., 2003), and sweet potato supplemented with yeast extract (Hayek et al., 2013). We believe that dates are a good substitute source of carbon, nitrogen, vitamins, and minerals and could be used in a low-cost media for the production of LAB.

Date palm (*Phoenix dactylifera L.*) is a rich source of carbohydrates, such as sucrose, glucose, and fructose, and proteins. Dates are good source of fiber and contain vitamins and minerals, including significant amounts of calcium, iron, fluorine, selenium, while being low in sodium (Tang, Shi, & Aleid, 2013). The date industry generates a huge amount of unwanted by-products. We believed that, instead of going to waste, these by-products could be taken advantage of to produce low-cost LAB media. The objective of this study was to investigate the feasibility of using date palm extract to cultivate *Lactobacillus reuteri*.

2. Materials and Methods

2.1 Preparing the Date Extract

Fresh date palm was obtained from local stores at Greensboro, NC. After removing the seeds, the date flesh was pressed for 6-7 days to release the date syrup. The date fiber was mixed with DDW at a ratio 1:2 (w/v). The mixture was stirred for 1-2 h and then incubated in a water bath at 65 ℃ for 2 h. The solution was blended for 5 min in a kitchen blender and soaked overnight at 4 ℃. The mixture was centrifuged at 4696 x *g* for 25 min at 4 ℃. The supernatant (1L) was collected and denominated date palm extract (DPE). The DPE was autoclaved at 110 ℃ for 15 min and stored at 4 ℃.

2.2 Preparing the Buffer Solution

Buffer Solution (BS) was prepared by dissolving the following reagents into 1L of diH_20: 1 g L-Cysteine.HCL, 2 g disodium phosphate Na_2HPO_4, 2 g ammonium citrate $NH_4C_6H_5O_7$, 5 g Sodium acetate $C_2H_3NaO_2$, 0.15 g Calcium chloride $CaCl_2$, 2 g Dipotassium phosphate K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.05 g $MnSO_4.5H_2O$, and 1 mL Tween 80 in 1 L. The mixture was autoclaved at 121 ℃ for 15 min.

2.3 Preparing the Date Palm Medium

Date palm medium (DPM) was prepared by mixing BS with DPE at a ratio of 2:3 (v/v). DPM was adjusted with a phytone peptone solution to a final concentration of 0.8 % (w/v). The media was dispensed into sterile 50 mL test tubes for experimental work.

2.4 Preparing the MRS

MRS broth was prepared by dissolving 55g MRS (deMan Rogosa Sharp, Neogen, Lansing, MI) broth and 1.0 g L-Cysteine. HCL into 1 L of diH_20 (Ibrahim, 2005). The broth was autoclaved at 121 ℃ for 15 min, cooled down to 48 ℃ in a water bath, and stored at 4 ℃. The media was used within 2 days.

2.5 Bacterial Culture Activation and Preparation

The growth of three strains of *Lactobacillus reuteri* were tested with the media: CF2-7F, DSM 20016 and SD 2112. These strains were obtained from the collections of the Food Microbiology and Biotechnology Laboratory at the North Carolina Agricultural and Technical States University. 100 µL cultures were transferred to 10 mL of fresh MRS broth. This was incubated at 37 ℃ for 24 h. Cultures were streaked onto MRS agar, incubated at 37 ℃ for 48 h, and stored at 4 ℃. A single colony of each strain was activated in 10 mL MRS broth and incubated at 37 ℃ for 16-18 h.

2.6 Adaption Proceeding

Overnight grown strains were individually sub-cultured twice in batches of 10 mL DPM broth and 10 mL of MRS broth, and then incubated for 24 h at 37 ℃. The activated strains were harvested by centrifugation at 4696 x *g* for 10 min at 4 ℃ and washed twice with 0.1% sterilized phosphate-buffered saline or PBS (pH 6.8).

2.7 Inoculation Procedure

Individual strains of *Lactobacillus* were serially diluted in PBS. Batches of DPM and MRS broth were inoculated with 100 µL of a bacterial dilution, corresponding to about 3.1 log CFU/ml. This was mixed thoroughly. Each batch was incubated at 37 ℃ for 24 h. The initial bacterial population was determined using the

bacterial enumeration method described below.

2.8 Measuring Bacterial Growth, TA, and Ph.

Bacterial growth was monitored by observing the turbidity. The turbidity was observed by measuring the optical density (O.D.) at 610 nm in a Genesys every two hours for 18 hours in 10S UV-V is spectrophotometer (Thermo Fisher Scientific, Madison, WI). The bacterial population, titratable acidity (TA), and pH were measured at 0h and 18 h.

The titratable acidity (TA) was determined by titrating the culture with 0.1 N NaOH while measuring the pH with a Accumet Basic pH meter (Fisher Scientific, Pittsburgh, PA). The TA was calculated as a percentage of acid. The pH of each sample was measured at the beginning (0 h) and end (18 h) of the fermentation period.

2.9 Bacterial Enumeration

Bacterial populations (log CFU/mL) at 0h and 18 h were determined by plating culture samples onto MRS agar. 1 mL samples were first serially diluted in 9 mL of PBS. 100 µL from the appropriate dilution were then surface plated onto MRS plates, in triplicate. Plates was incubated anaerobically at 37 ℃ for 48 h. Plates with 25-250 colonies were counted and the results were converted to log CFU/mL.

2.10 Statistical Analysis

Each experiment was independently replicated 3 times. The analysis and enumeration were carried out in duplicate. The mean square of each treatment was calculated. Duncan's multiple range test was used to find significant differences. A difference between treatments was considered significant when a P value was calculated to be less than 0.05. The experiment was carried out using a randomized design. Using SAS v9.2, an analysis of variance was carried out to determine significant effects at the significance level of $P < 0.05$.

3. Results

3.1 Preliminary Study

Various factors could be applied to improve the nutritional capacity of LAB growth media (Nancib et al., 2001). Here, we conducted several preliminary experiments to determine if date palm extracts could support the growth of *Lactobacillus reuteri*. We tested many conditions including: different date fiber to water at ratios, soaking regimes, and heating temperatures. The date fiber from the pressed date fruit was mixed with deionized distilled water (DDW) at different concentrations (10, 20, and 50 %, w/v). Each solution was kept overnight at 4 ℃ for use the next day. We supplemented the nitrogen source of half the DPM with phytone peptone (PP). As shown in Table 1, the DSM 20016 strain of *Lactobacillus reuteri* strain did not grow well in 10% DPM broth compared to MRS. Moreover, the growth of *L. reuteri* DSM 20016 in 20% DPM was lower than in MRS over an 18 h incubation at 37 ℃. However, DSM 20016 did grow substantially in 50% DPM, reaching an $O.D._{600}$ of 0.562. These results proved that DPM can support the growth of LAB. Furthermore, when DSM 20016 was grown in 50 % DPM with 0.8 % Phytone Peptone (PP), a significantly higher O.D. of 0.762 was achieved. This demonstrates that DPM has the potential to promote the growth of *Lactobacillus* spp.

Table 1. Growth of DSM 20016 during an incubation in MRS and DPM for 18h at 37 ℃. Growth was monitored by measuring optical density at 610 nm.

Growth Mediu	DPM (%)	Incubation Time (hr)	
		0	18
		Optical density (OD 610 nm)	
MRS		0.031	1.52
DPM	10	0.006	0.016
	20	0.016	0.312
	50	0.018	0.562
	50+0.8 % PP	0.019	0.762

PP = Phytone Peptone

3.2 Date Palm Extract Supplemented With Phytone Peptone (PP)

Figure 1 (A-C) shows the growth of three strains of *Lactobacillus reuteri*, DSM 20016, CF2-7F, and SD 2112, in MRS and DPM broth. Growth was monitored by measuring the optical density at 610 (O.D. 610). In MRS medium, the O.D. 610 reached 0.897 on average during 8 h of incubation at 37 ℃. When *L. reuteri* were grown in the DPM control there was no visual growth. When DPM (50 %) was supplemented with 0.2 % of phytone peptone (PP) the DSM 20016 strain grew, reaching an O.D. 610 of 0.35 after 8 hr. At 0.4 %, 0.6%, and 0.8% PP, the O.D. 610 reached 0.48, 0.63, and 0.77, respectively. Similar growth patterns were observed with the CF2-7F and SD 2112 strains. These results showed that the growth of *L. reuteri* is dependent on the concentration of PP

and not on the strain.

Table 2 lists the bacterial populations, expressed as log CFU/mL, of each *L. reuteri* strain after an18 h incubation at 37 ℃. DSM 20016 strain continued to grow in MRS, crossing from an initial population 2.55 log CFU/mL and reaching 7.86 Log CFU/mL. Meanwhile, in the DPM, less PP, DSM 20016 reached a bacterial population of only 3.54 Log CFU/mL from an initial population of approximately 2.5 log CFU/mL. When DPM was supplemented with 0.2%, 0.4%, and 0.6%, and 0.8% PP the population reached 4.55, 4.65, 6.63, and 7 log CFU/mL, respectively. Similar results were seen with CF2-7F and SD 2112.

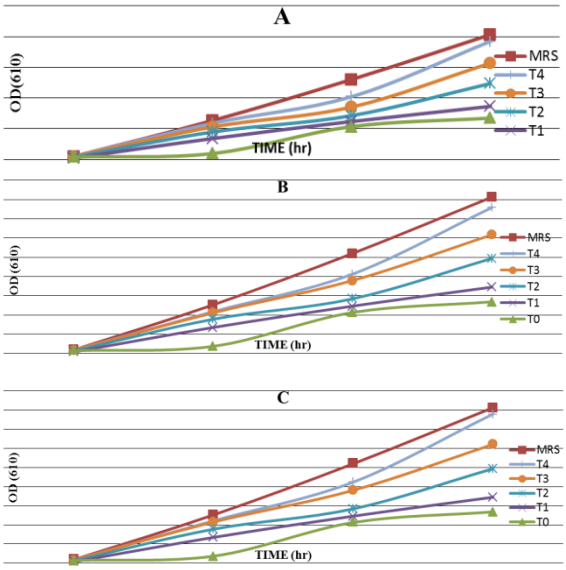

Figure 1. Growth of DSM 20016 (a), CF2-7F (b), and SD 2112 (c) during an incubation at 37 ℃ for 8 h in MRS and DPM, as measured by the optical density at 610 nm (T0= control, T1= 0.2 %, T2= 0.4 %, T3= 0.6 %, T4= 0.8 % of Phytone Peptone = PP)

Table 2. Bacterial Populations of DSM 20016, CF2-7F, and SD 2112 during an incubation at 37 ℃ for 18 h in MRS and DPM.

Growth Medium	*Lactobacillus reuteri* Strains		
	DSM 20016	CF2-7F	SD 2112
MRS (Standard)	7.89 ± 0.02	7.84 ± 0.02	7.86 ± 0.01
CONTROL	3.54 ± 0.1	3.55 ± 0.1	3.57 ± 0.1
DPM+0.2PP	4.55 ± 0.0	4.48 ± 0.0	3.51 ± 0.0
DPM+0.4PP	4.65 ± 0.1	4.52 ± 0.1	4.34 ± 0.0
DPM+0.6PP	6.63 ± 0.1	6.68 ± 0.0	6.69 ± 0.0
DPM+0.8PP	7.00 ± 0.1	6.93 ± 0.0	6.91 ± 0.0

Results are presented in log CFU/mL (T0= DPM control, T1= 0.2 %, T2= 0.4 %, T3= 0.6 %, T4= 0.8 %)

* Initial inoculum level was approximately 2.5 Log CFU/mL

4. Discussion

Previously, we found that phytone peptone (PP) is the most important growth-promoting factor for *Lactobacillus reuteri* (Atilola, Gyawali, Aljaloud, & Ibrahim, 2015). Our results indicate that DPM supplemented with PP support the growth of *L. reuteri*. Earlier results also showed that date palm extract supplemented with external nitrogen sources can support lactobacilli growth (Nancib et al., 2001). Our results also agree with the work of Hayak et al. (Hayek et al., 2013) who observed that *L. reuteri* grew well in sweet potato based medium supplemented with a nitrogen source. Date palm is a rich source of a variety of nutrients and has the potential to either partly or fully replace the expensive ingredients of LAB media. Here we show that we could not only replace the carbon source, but could also greatly reduce the use of added sources of nitrogen. Standard MRS uses a total concentration of nitrogen sources of around 2.5% (per Liter, 10g protease peptone #3, 10g of Beef extract). Using DPM, that amount can be reduced to 0.8% using the more economical soy-based phytone peptone (see Table 3). Therefore, we have shown that DPM is more economical than standard MRS. There was no significant ($P > 0.05$) difference in the growth of LAB in MRS and DPM medium developed. These results demonstrate that DPM + 0.8 PP could be used as an alternative low cost medium.

Table 3. The total cost of nitrogen sources according to purchases made from BD BioSciences.

Media	Nitrogen Sources (g)	Cost per Gram	Total Cost of Nitrogen Sources per Liter of Media
MRS	Proteose peptone #3 (10g)	\$0.467/g	\$4.67
	Beef Extract (10g)	\$0.358/g	\$3.58
	Yeast extract (5g)	\$0.194/g	\$1.94
			Total \$10.19
DPM	Phytone peptone (8g)	\$0.186/g	Total \$1.49

5. Conclusion

DPM + 0.8% PP could be a suitable low-cost medium for the growth of LAB, in particular, strains of *Lactobacillus reuteri*. These findings might lead to more interest in the applications of date palm by-products in lactic acid fermentation.

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