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Production of Lactic Acid by a Local Isolate of *Lactobacillus plantarum* Using Cheap Starchy Material Hydrolysates

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Abstract: Some cheap starchy materials like sorghum grains and wheat bran were degraded by crude glucoamylase of a local isolate of *Mucor* sp., then Lactic Acid (LA) was produced by a local isolate of *L. plantarum* using sorghum flour hydrolysate, sorghum starch hydrolysate, soluble starch hydrolysate, wheat bran hydrolysate and date syrup with 10% reducing sugars. The yield of LA increased to 37.2 g/100 reducing sugars by using sorghum flour hydrolysate as a basal medium supplemented with (0.6+0.6)% yeast extract+$(NH_4)_2HPO_4$ and 0.06% $MgSO_4.7H_2O$, reducing sugars was 5%. The fermentation temperature was 30°C/96 h. Results indicated that using hydrolysates mixtures of sorghum flour and wheat bran improved LA fermentation. The yield of LA was 92.5 g by using sorghum flour and 50% wheat bran. Paper chromatography indicated that LA was the unique organic acid in the fermented broth.

Key words: Lactic acid bacteria, *L. plantarum*, lactic acid, glucoamylase, fermentation

INTRODUCTION

Lactic acid is one of the earliest organic acids known, it was first discovered in 1780 by Scheele (Adthalungrong and Temviriyanukul, 2010). (LA) can be used as a preservative, acidulant and flavor in food, textile and pharmaceutical industries. It could become a commodity chemical for the production of lactate esters propylene glycol, propylene oxide, acrylic acid, 2,3-pentauedione, propionic acid aldehyde and dilactide (Dumbrepatil *et al*., 2008). It has also been increasing in importance as a feedstock for manufacture of Polylactic Acid (PLA) which could be a good substitute for synthetic plastic derived from petroleum feedstock. Approximately 90% of the total lactic acid produced worldwide is by bacterial fermentation (Zhou *et al*., 2006).

Although the production of (LA) is possible via chemical synthesis and biotechnology, current tendency of commercial scale (LA) production relies more on microbial fermentation of various renewable resources since it causes less environmental pollution (Wee *et al*., 2006).

In recent years, fermentation approach has become more successful because of increasing market demand for naturally produced (LA) with an estimated worldwide demand 130,000-150,000 ton/year (Panda and Ray, 2008). Another major advantage of fermentative lactic acid production is that stereo-specific acid can be achieved by a selection of appropriate microbial strains, chemical synthesis yields a racemic mixture form (Hofvendahl and Hahn-Hagerdal, 2000; Narayanan *et al*., 2004). It means that chemical synthetic only produce racemic mixture of L(+) and D(-) enantiomers but fermentation has added advantage of producing biologically active L(+) or D(-) or DL form of (LA) (John *et al*., 2005; John *et al*., 2006).

Most studies within production of (LA) have focused on the use of pure substances such as glucose (Kwon *et al*., 2001) or lactose (Amrane and Prigent, 1996) for the production. The use of natural substrates like starch (Altaf *et al*., 2006; Ohkouchi and Inoue, 2006) or cellulose (Adsul *et al*., 2007; Venkatesh, 1997) is economically unfavorable because they are very expensive and also require pretreatment in order to release for mentable sugars.

(LA) bacteria typically have complex nutritional requirements, due to their own growth factors such as B vitamins and amino acids. They require some elements, in the yeast extract for growth, such as carbon and nitrogen sources in the form of carbohydrates, amino acids, vitamins and minerals (Wee *et al*., 2006).

L. plantarum was used as a starter culture because it is homofermentative [produce only (L+) LA but not ethyl alcohol] and is a probiotic that confers various health beneficial effects to consumers (Montet *et al*., 2006) and frequently encountered in human gastrointestinal tract (Kleerebezem *et al*., 2003).

Hence, in the present study attempts were made to obtain maximum yields of (LA) using an alternative nutrients like wheat bran and sorghum flour for (LA) fermentation. Attempts were also made to increase the reducing sugars content of substrates used for fermentation by using crude fungal glucoamylase to obtain starch hydrolysate.

MATERIALS AND METHODS

Glucose and soluble starch from BDH chemicals, MRS-agar from Hi-media, sorghum grains, date syrup and wheat bran from the local markets in Basrah city, crude glucoamylase was produced by a local isolate of *Mucor* sp. and a local isolate of *Lactobacillus plantarum* were obtained from Department of Food Science, College of Agriculture, University of Basrah.

Estimation of reducing sugars by using 3, 5-dinitrosalcylic acid according to Miller (1959). Glucose was used as a standard reducing sugar and absorbance was at 550 nm.

Starch extraction from sorghum grains was according to Yang and Seib (1995) with modification: Grain sorghum (100 g) as a dry weight and water (150 ml) were ground for 1 min at low speed (18,000 rpm), then for 2 min at high speed (22,000 rpm) in a blender. The slurry, with temperature of ≈38°C was poured on an 80 wire mesh screen and the overs were washed with water (3 x 50 ml). The throughs then obtained by using two screen, the upper was 75 µm (200 wire mesh) and the lower was 40 µm (400 mesh), then throughs were washed with water (1 x 30 ml) and centrifugation with 12,000 x g/20 min. The upper layer of precipitate was discarded then mixed with water (30 ml), then centrifugation repeated twice and precipitated starch was dried in oven at 40°C/24 h.

Degradation of starchy materials by glucoamylase: Each of 20 gm of sorghum flour, sorghum starch, soluble starch and wheat bran were in beakers and 80 ml of dist. water were added to each beaker to make suspention of 20% (w/v), pH was adjusted to 6 by using 0.1N HCl, then glucoamylase was added. After dilution, the mixtures were left on 60°C/30 min, the reaction was stopped on 85°C/10 min. The hydrolysate was filtered through nylon cloth and 80 ml filtrate was collected by using additional quantity of water for washing the residue. Clear hydrolysate was obtained by filtering the samole through filter paper (Whatman no.1).

Bacterial inoculum preparation: The inoculum was prepared by subculturing *L. plantarum* in skim milk (10%) and incubated at 37°C/24 h.

Optimum conditions of lactic acid production: Many conditions had been studied for the optimum production of lactic acid by *L. plantarum*, they were: The nutrients, temperature, pH and incubation period.

The media were prepared in 100 ml volumes by diluting the hydrolysates of sorghum flour, sorghum starch, wheat bran and soluble starch individually, date syrup was also used after diluting with water. The reducing sugars were adjusted to 10% (w/v) and pH to 6.0, sterilized in 250 ml conical flasks by autoclave then added inoculum (1%) and incubated at 37°C/96 h (Shamala and Sreekantiah, 1988).

The temperature: (25, 30, 35, 40)°C.

The pH: (5.0, 5.5, 6.0, 6.5).

The incubation period: (24, 48, 72, 96, 120) h.

The nutrients%: $(NH_4)_2HPO_4$ (0.2, 0.6, 0.8), yeast extract (0.2, 0.6, 0.8), $MgSO_4.7H_2O$ (0.02, 0.06, 0.08), yeast extract+$(NH_4)_2HPO_4$ (0.2+0.2), (0.2+0.6), (0.6+0.2), (0.6+0.6), respectively.

Reducing sugars: (2, 5, 10, 15)%.

Lactic acid was precipitated as calcium lactate, then separated from the broth, dried at 50°C and weighed.

$Ca(OH)_2$ solution 0.1N was used in adjusting the pH of the media to 6.0 during fermentation and the reducing sugars and total acidity were estimated every 24 h of fermentation. The percentage of lactic acid was determined as (g) produced from 100g reducing sugars. Lactic acid was identified by paper chromatography using filter paper (Whatman no.1) and the solvent system was n-butanol: formic acid (10: 2) with bromocresol green 0.1% (w/v) as indicator. Pure lactic acid was used as a standard. After air drying, the paper was exposed lightly to ammonia vapors, lactic acid appeared as orange spot on a blue background (Shamala and Sreekantiah, 1987).

Table 1: The chemical composition of some carbon sources

Sample	M	P	F	C	Ash
	(%)				
Sorghum flour	7.1	13.6	3.4	74	1.9
Wheat bran	14.3	14.0	0.0	70	1.7
Date syrup	20.1	0.0	0.2	72	7.7

M = Moisture (%), P = Protein (%), F = Fat (%), C = Carbohydrate (%)

Table 2: Reducing sugars in the starchy substrates after hydrolyzing by crude glucoamylase

Hydrolysate	Reducing sugars (g/100 g carbohydrate)
Sorghum flour	87
Sorghum starch	96
Soluble starch	91
Wheat bran	25
Date syrup	52

RESULTS AND DISCUSSION

The chemical composition of the sorghum flour, wheat bran and date syrup are shown in Table 1.

Most Lactobacilli do not readily metabolize starch (Rogosa, 1974), in an attempt to utilize starchy substrates for lactic acid fermentation, enzyme hydrolyzed materials were tried (Table 2).

The results showed that the starchy substrates contained high quantities of reducing sugars after hydrolysis by glucoamylase especially sorghum starch and soluble starch, it means that the glucoamylase activity increased with using these both materials (each of them) as a substrate because of their purity but sorghum flour and wheat bran had other contents in addition to starch, so they might have an effect on glucoamylase activity. The inoculum of *L. plantarum*

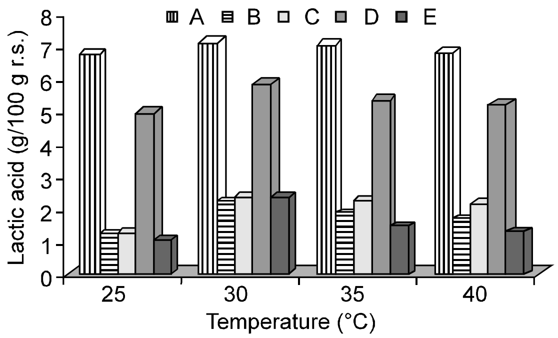

Fig. 1: Effect of temperature on (LA) production by a local isolate of *L. plantarum* using different starchy hydrolysate and date syrup. A = Sorghum flour h, B = Sorghum starch h, C = Soluble starch h, D = Wheat bran h, E = Date syrup

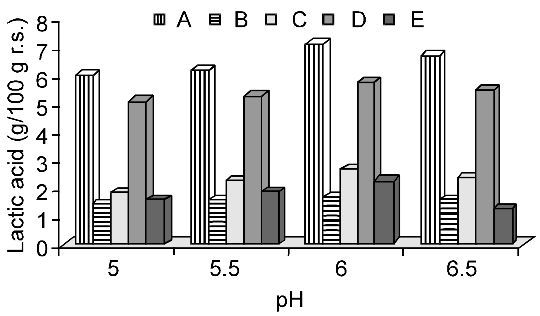

Fig. 2: Effect of pH on (LA) production by a local isolate of *L. plantarum* using different starchy hydrolysates and date syrup. A = Sorghum flour h, B = Sorghum starch h, C = Soluble starch h, D = Wheat bran h, E = Date syrup

(1%) contained 9.2 x 10^8 c.f.u/ml. Optimum temperature of lactic acid fermentation by *L. plantarum* using starchy hydrolysates and date syrup was shown in Fig. 1.

Sorghum flour hydrolysate produced the highest quantity of (LA) (7.2 g/100 g reducing sugars) at 30°C, it means that 30°C was the suitable temperature for *L. plantarum* to produce (LA) (Shamala and Sreekantiah, 1987; Shamala and Sreekantiah, 1988). From Fig. 2 it is observed that the optimum pH was 6.0 for (LA) production by using sorghum flour hydrolysate and the yield was 7.1 g. The results were agreed with Ohkouchi and Inoue (2006) and Dumbrepatil *et al.* (2008).

The yield of lactic acid was 8.5 g after fermentation of sorghum flour hydrolysate for 96 h (Fig. 3).

The optimum conc. of yeast extract and $(NH_4)_2HPO_4$ was 0.6% as shown in Fig. 4 and Fig. 5. These figures gave 16.0 g and 11.7 g (LA) respectively using sorghum flour hydrolysate. Yeast extract has long been reported to be a good source for (LA) fermentation, it is rich in amino acids, vitamin B groups and nitrogen for growth and other growth factors required for (LA) bacteria

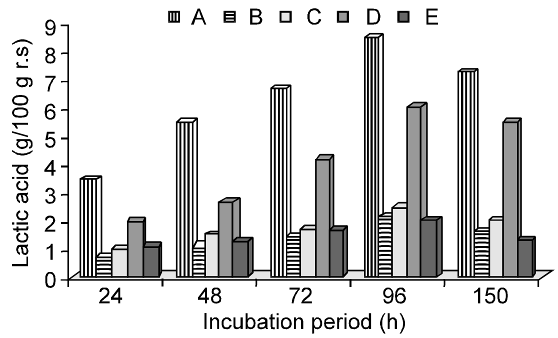

Fig. 3: Effect of incubation period on (LA) production by a local isolate of *L. plantarum* using different starchy hydrolysates and date syrup. A = Sorghum flour h, B = Sorghum starch h, C = Soluble starch h, D = Wheat bran h, E = Date syrup

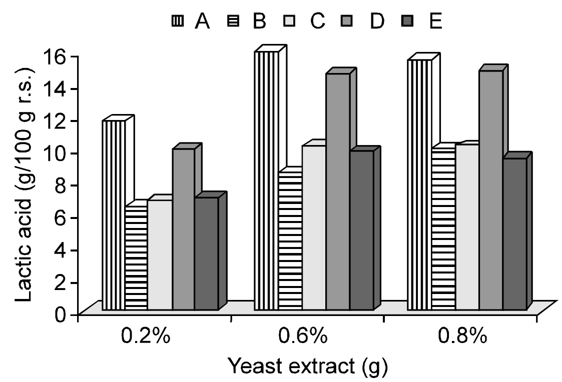

Fig. 4: Effect of adding of yeast extract to the basal media on (LA) production by a local isolate of *L. plantarum* using different starchy hydrolysates and date syrup. A = Sorghum flour h, B = Sorghum starch h, C = Soluble starch h, D = Wheat bran h, E = Date syrup

(Fitzpatrrick *et al.*, 2001). Ammonium phosphate is a good inorganic source of nitrogen for growth and (LA) production and at the same time plays as a buffering factor to control pH decreasing. The nutrients such as yeast extract, $(NH_4)_2HPO_4$ and $MgSO_4.7H_2O$ increased the efficiency of (LA) fermentation according to Aksu and Kutsal (1986), also Qi *et al.* (2009) observed that yeast extract could be decreased from 1.0% to 0.7% (w/v) with adding 0.07 (w/v) tween-80, while still maintaining high (LA) yield.

The results agree with Adthalungrong and Temviriyanukul (2010), they optimized (LA) production by using yeast extract, but its quantity was 2%. Shamala and Sreekantiah (1988) got the same results obtained in this study, they obtained high production of (LA) at 0.5% for each yeast extract and $(NH_4)_2HPO_4$. However $MgSO_4.7H_2O$ has no effect on (LA) production. Our results showed that at 0.06% of $MgSO_4.7H_2O$, (LA) production increased to 7.7 g (Fig. 6).

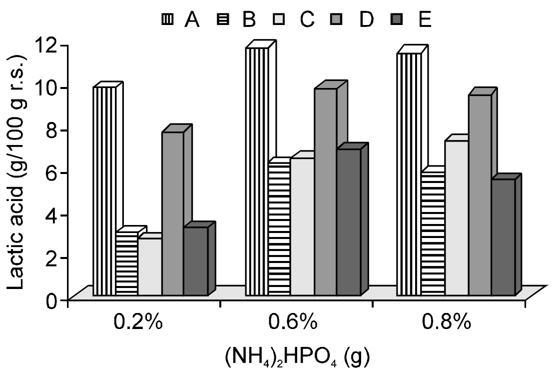

Fig. 5: Effect of adding of $(NH_4)_2$ HPO_4 to the basal media on (LA) production by a local isolate of *L. plantarum* using different starchy hydrolysates and date syrup. A = Sorghum flour h, B = Sorghum starch h, C = Soluble starch h, D = Wheat bran h, E = Date syrup

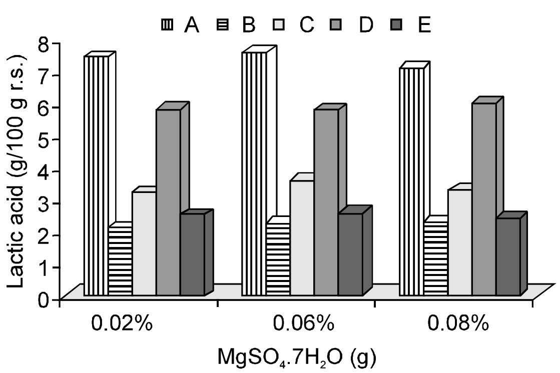

Fig. 6: Effect of adding of $MgSO_4.7H_2O$ to the basal media on (LA) production by a local isolate of *L. plantarum* using different starchy hydrolysates and date syrup. A = Sorghum flour h, B = Sorghum starch h, C = Soluble starch h, D = Wheat bran h, E = Date syrup

Table 3: Effect of percentage of wheat bran hydrolysate which mixed with sorghum flour hydrolysate with 5% reducing sugars on (LA) production by a local isolate of *L. plantarum* using different starchy hydrolysate and date syrup

Starchy substance hydrolysates	Lactic acid
Sorghum flour + 10%wheat bran	48.0%
Sorghum flour + 30%wheat bran	65.0%
Sorghum flour + 50%wheat bran	92.5%

The experiment of adding both yeast extract and $(NH_4)_2HPO_4$ to the basal media observed that (0.6+0.6) g/l was the best; it gave 22.5 g (LA) using sorghum flour hydrolysate (Fig. 7).

Reducing sugars were important in (LA) fermentation, so controlling to 5% increased the yield to 37.2 g using sorghum flour hydrolysate (Fig. 8).

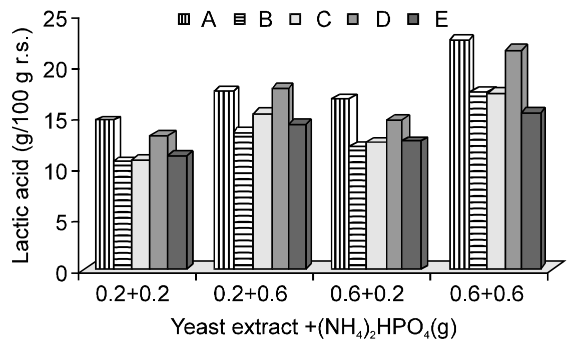

Fig. 7: Effect of adding each of yeast extract + $(NH_4)_2$ HPO_4 to the basal media on (LA) production by a local isolate of *L. plantarum* using different starchy hydrolysate and date syrup. A = Sorghum flour h, B = Sorghum starch h, C = Soluble starch h, D = Wheat bran h, E = Date syrup

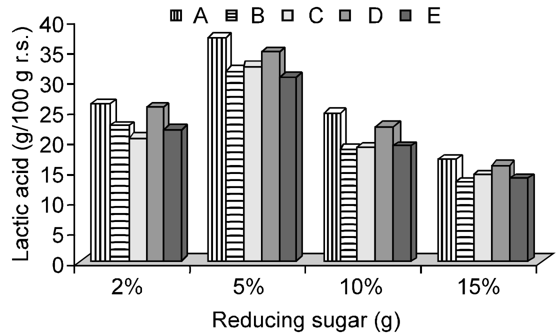

Fig. 8: Effect of reducing sugars (%) in the fermentation broth on (LA) production by a local isolate of *L. plantarum* using different starchy hydrolysates and date syrup. A = Sorghum flour h, B = Sorghum starch h, C = Soluble starch h, D = Wheat bran h, E = Date syrup

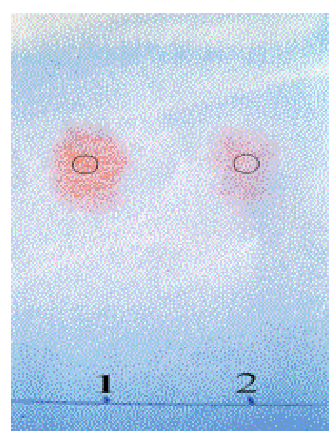

Fig. 9: Identification of lactic acid by paper chromatography. 1 - Pure lactic acid; 2 - Produced lactic acid

In all experiments, the yield of (LA) was higher with sorghum flour hydrolysate. In addition to the nutrients the flour also contains protein which may also help the prevention of a rapid drop in the pH of fermentation broth (Shamala and Sreekantiah, 1988). To improve the fermentation process we mixed sorghum flour hydrolysate of 5% reducing sugars with 50% wheat bran hydrolysate, the yield of (LA) was increased to 92.5 g (Table 3).
Paper chromatography was to detect (LA) by using pure (LA) as shown in Fig. 9, there was one spot at the same line with the pure, this means (LA) was the unique organic acid which produced by *L. plantarum*.

Conclusion: The results indicated that there are many cheaper and available materials can be utilized for (LA) fermentation like sorghum grains and wheat bran, because they are good sources of carbon and nitrogen for growth of (LA) bacteria and production of (LA). Also these materials contain protein which can increase the yield of (LA) by prevention a rapid drop in the pH of the medium. By other way, the starchy substrates like soluble starch and sorghum starch without supplementation were not suitable media because they were carbon sources only. So they did not enhance the growth of (LA) bacteria. Also the date syrup was carbon source with no nitrogen, to therefor, this medium enhanced (LA) production at the early stage of fermentation and the rapid decreasing of pH inhibited the growth of bacteria. This is an indication that date syrup was a poor medium for (LA) production.

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