THE PRODUCTION OF LACTIC ACID BY THE LOCAL IRAQI STRAIN *ENTEROCOCCUS FAECIUM*

Maytham A. Alhamdani* Amal Kadhim Al Asady**

Wissal Audah Hassan Alhilfi***

*Department of Biology, College of education for pure science, University of Basrah, Basrah,Iraq.

**Department of Biotechnology, College of Agriculture, University of Basrah Basrah,Iraq.

*** Department of Biology, College of science, University of Basrah Basrah,Iraq.

(Received 25 February 2014 ,Accepted 6 May 2014)

Key words: *Enterococcus*, Lactic acid, Whey.

ABSTRACT

Lactic acid is an important carboxylic acid used in different aspects of life. In this study a pure isolate of homofermentative lactic acid bacteria *Enterococcus faecium* was isolated , and characterized by morphological examinations with physiological and biochemical tests.

The present results have revealed the ability of the isolate to produce L- lactic acid in high concentration and low concentration of D- lactic acid in contrast with control. Lactic acid has been extracted and partially purified by Amberlite IRA400 resin column, the obtained extracted parts have been tested by carboxylic acid test in addition to High Performance Liquid Chromatography (HPLC) analysis which has showed that the concentration of L-lactic acid was 0.659g/l, while D-lactic acid was 0.174g/l in crude parts with two peaks appeared, one of area 322854 for L while no visible peak appeared for D because of small area 72214 in the figure, whereas the second peak of area 203238 may returned to the interference of D and L-lactic acid . Purified parts have reached to a purity of 72.3% for L-lactic and 38.2% for D-lactic acid also with one sharp peak for L with area of 43639 and no visible peak for D-lactic due to small area of 16713.

INTRODUCTION

Lactic acid (2-hydroxypropanoic acid) is the simplest 2-hydroxy carboxylic acid (or α-hydroxy acid) with a chiral carbon atom and exists in two enantiomeric forms (1). It exists in two different forms the *dextrorotatory* form, called L(+)-lactic acid or (*S*)-lactic acid, and the *levorotatory* form, called D(−)-lactic acid or (*R*)- lactic acid. The plus and minus signs indicate the direction of the rotation of plane polarized light produced by a chemical. These 2 stereoisomers (scientifically known as "enantiomers") are produced by different enzymes [lactate dehydrogenases (LDH)] present in living organisms. Naturally formed lactic acid is usually in the L form, but D-LA may coexist with L-LA in some cases, especially if it is secreted by nonspecific microbes (2).

Many members of the order *Lactobacillales* produce lactic acid as their major or sole fermentation product and are sometimes collectively called **lactic acid bacteria.** *Streptococcus, Enterococcus, Lactococcus, Lactobacillus,* and *Leuconostoc* are all members of this group (3).

Lactic acid production was produced in five selected rumen strains of *Enterococcus faecium* (4). *Enterococcus faecium* was isolated from *Puto* a type of fermented rice in the Philippines (5) and used to produce lactic acid in repeated batch fermentation mode.

This study has aimed to isolation and identification of homofermentative lactic acid bacterium, with production and purification of lactic acid in whey medium.

MATERIALS AND METHODS

Isolation and cultivation of bacteria

The media M17 broth (Himedia, India) and de Man Rogosa Sharpe (MRS) agar (Oxoid, England) have been prepared according to the instructions of manufacturing companies as possible, with addition of approximately 1.5- 2g /100 ml agar to M17 broth for prepairing M17 agar. All media have been sterilized by autoclave adjusted at 121°C, 15- 20 min. and 0.15 Mpa. A loopful of raw animal milk sample have been cultured on M17 and/ or MRS plates, left in the incubator adjusted at 30°C± 1for 24- 48 or 72 hr. (6) with modifications.

Different colonies have been sub cultured by streaking for purification (7).

Morphological characterization

Selected colonies have been characterized on M17 agar, by recognizing of shape, color, consistency and size. Whereas Gram staining (Arcomex, Jorden) has been applied for selected isolate by following the manufacturing company information as possible.

Biochemical tests

Catalase test slide technique (8), clotting test (6) and gas production test (9) have been done with some alterations, while Strepto- system 9R test (Liofilchem, Italy) has been processed by following the instruction of manufacture company as possible.

Physiological identification

Growth at temperatures of 10 and 45 °C, growth at NaCl concentration of 4 and 6.5% and growth at pH of 9.6 have been done (10), with some modifications (incubation 24- 48hr., 0.1ml inoculum and 620nm wave length).

Production, extraction and purification of lactic acid

Homemade Whey from animal source has been passed through a piece of cloth, dispensed into conical flasks about 50 ml whey for each, sterilized by autoclave, adjusted to 113°C for 10 min and pH adjusted to 6±0.2 by one of them with about 1N of NaOH, and put the same amount of NaOH in the others without measuring pH, then inoculated with about 0.5ml of approximately 24 hr. bacterial culture, left in the incubator adjusted at 30°C± 1for 24- 48hr.

Flasks have been heated in the water path at 90-97 °C for about 10±2 min., cooled , centrifuged at 7000- 8000 cycle/min. by table top centrifuge (Gemmy, Taiwan) for about 10 min., then filtered with filter paper type Wattman no. 1and milli pore filter paper (11) with some modifications. About 5ml each has been dried at 80- 85°C and prepared for analysis.

The previous production steps has been repeated to a single flask, with increased in inoculate volume to about 1ml and incubation period for 48- 72 hr. and then prepared for purification.

According to Cao *et al*., (12) and Tong *et al*., (13) with many modifications (column's dimension, acid and base Normailty and volumes, acid type and others); nearly 20 g Amberlite IRA 400 resin (BDH, England), has been soaked in a distilled water for about 24 hrs., filled up in about (1.6×71.5) cm column, conditioned with different column's volume (resin volume) with about (1N NaOH, D.W., 1N HCl, D.W., 0.1N NaOH, 0.1N HCl) alternately, and continuous randomly volumes until reach to pH

nearby 6. About 20 ml sample has been drained in the column, washed with40 ml water, then 60 ml approximately of HCl loaded in the column to get the acid, with trying to keep slow flow rate between 0.1 to 0.6 ml/min for HCl and sample as possible. Each 20 ml have been obtained in separate tube, prepared for analysis.

Chemical characterization of lactic acid.

According to (14) carboxylic acid test has been done and about 10ml of the purified sample has been dried and sent along with the non-purified samples to Green barrows laboratories/Baghdad to be analyzed by High performance liquid chromatography (HPLC), a Shimadzu binary pump model LC-20A with automatic sample injector and a RID 20A differential refractometer, copper(Π)acetate (2mmol/l) with or without water as eluent and flow rate of 1.0ml/ min. have been used.

RESULTS

Isolation and cultivation.

Suspected isolate has been selected from raw animal milk samples termed as B4.

Morphological characterization.

On M17 and MRS agar, colonies have appeared white, creamy, pin point to small colonies after 24hr. Staining has revealed that cells are Gram ositive, cocci to ovoid in pairs or medium chains, (Figure 1).

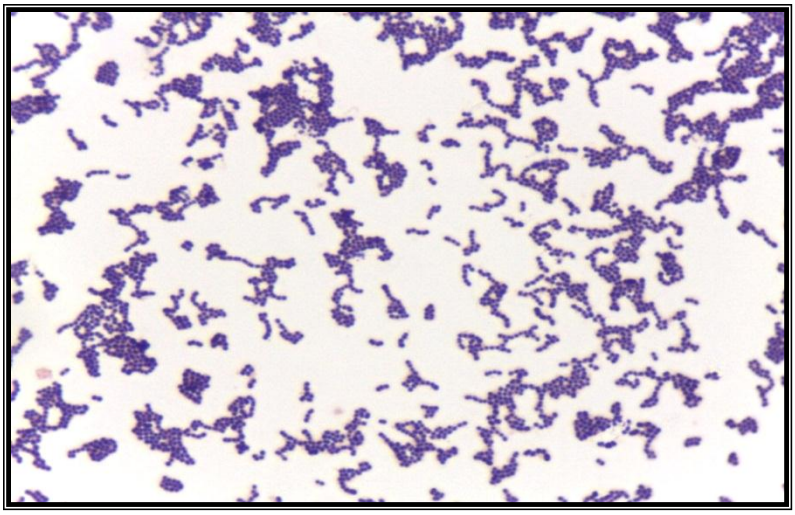

Figure (1) : Gram staining of B4 isolate, showed Gram positive short or medium chains Biochemical tests.

Isolate was negative to the catalase and no bubbles have been shown, whereas in the skim milk clot has been produced in both tubes in contrast with control, beside that no gas has been liberated neither in M17 nor MRS broth. In table (1) different reactions have been shown in Strepto- system 9R tests.

Table (1): Biochemical test showed the results of Strepto- system 9R test

Isolate / Test	B4
Pyroglutamic-β-Naphthylamide	-
Aesculin	V
Hippurate	V
O-Nitrophenyl-Beta-D-Galactopiranoside	+
Arabinose	-
Mannitol	-
Raffinose	-
Bacitracin	-
Optochine	-

(+) positive, (-) negative, (V) variable, B4: *Enterococcus faecium*

Physiological identification.

Study of growth under different temperatures, NaCl and pH has been compared in table (2).

Table (2): Growth of isolate *Enterococcus faecium* under different conditions

Isolate name / Growthcondition	B4
Temprature (°C)	
10	+
45	-
Salinity (NaCl%)	
4	+
6.5	V
pH	
9.6	V

(+): visible growth, (V): variable (sometimes visible growth another time no growth or one replicate contain growth and no in the other), and (-): growth ≤0.1 (no growth), B4: *Enterococcus faecium*

Chemical characterization of lactic acid.

Chemical analysis of crude extract.

Different concentrations of L and D- lactic acid have been found during HPLC analysis of the crude extract, table (3) and figure (2a).

Chemical analysis of purified lactic acid.

Many bubbles of CO_2 have been shown in the reaction of part 2 and 3, but not found in the part 1. In the HPLC analysis, results have shown sharp L- lactic acid peak in the purified extracted sample as in the figure (2b), with high purity percent in comparison with the D- lactic acid, table (3).

Table (3): HPLC results showed concentrations and purity of lactic acid in crude and purified extracted samples.

Isolate name	Lactic acid concentration (g/l)		Lactic acid concentration (%)		Lactic acid purity (%)	
	D	L	D	L	D	L
B4	0.174	0.659	18.27	81.72	38.2	72.3
Con	0.00	0.00	0.00	0.00	0.00	0.00

B4: *Enterococcus faecium* , Con: control.

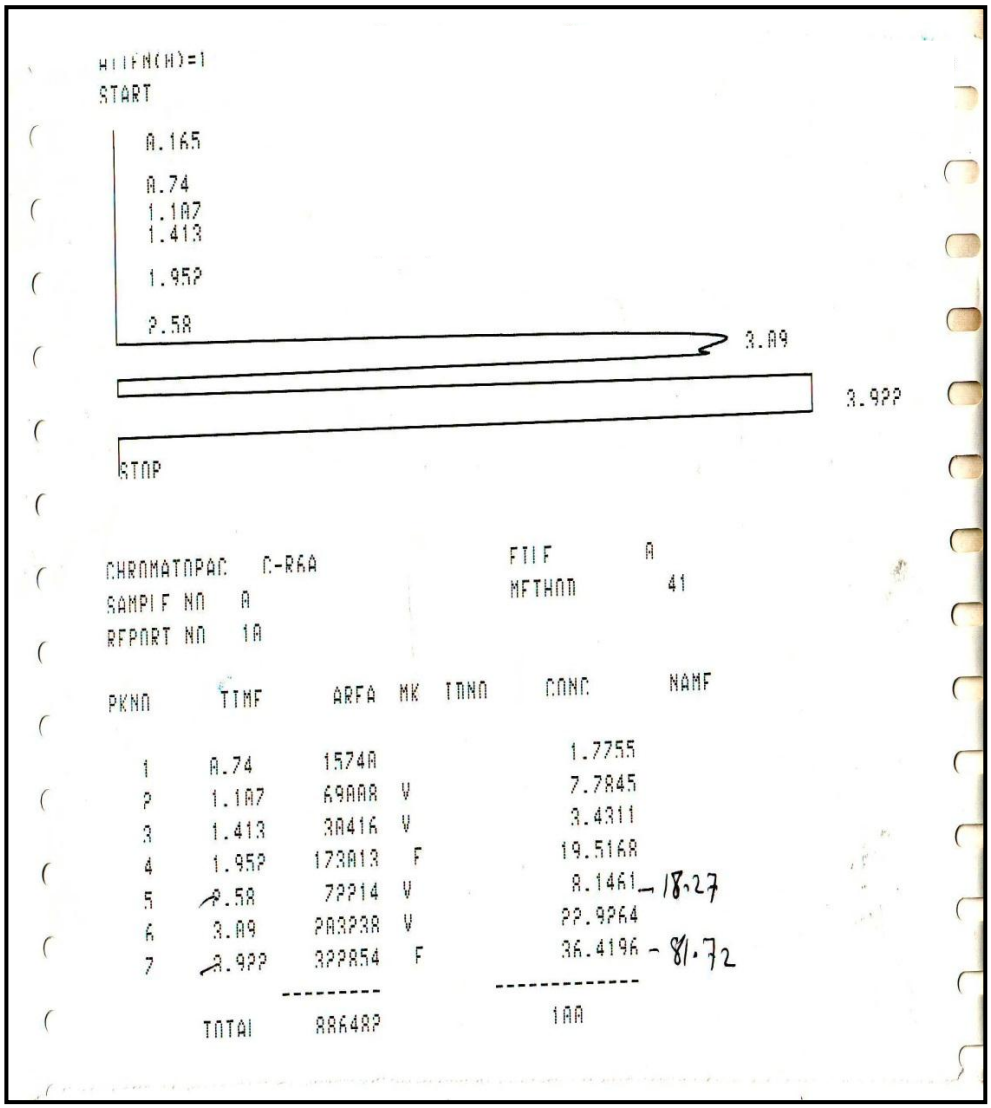

Figure (2a): HPLC analysis of crude extract of B4 inoculated whey

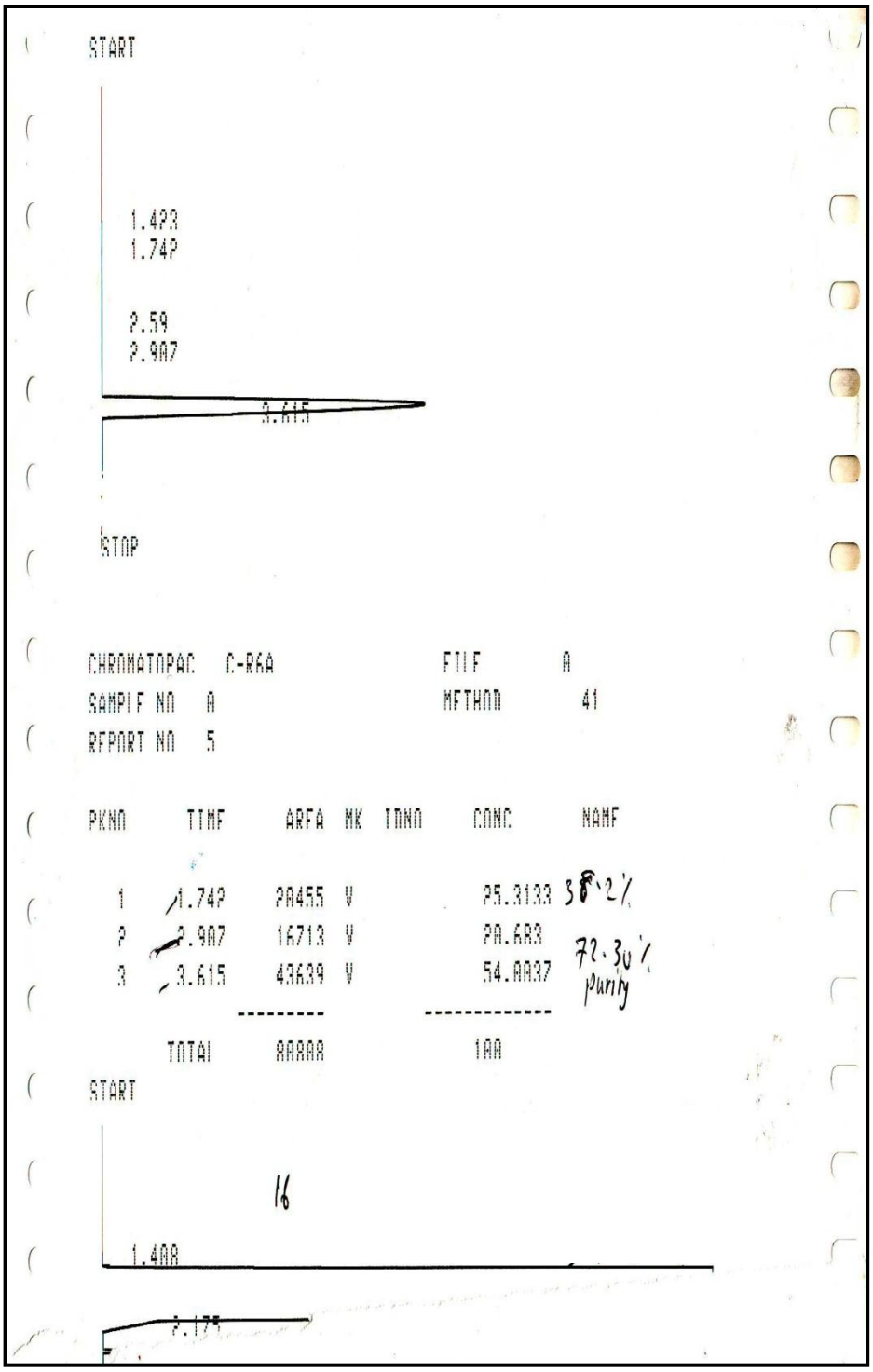

Figure (2b): HPLC analysis of purified extract of B4 inoculated whey

DISCUSSION

This study has focused on *Enterococcus faecium*; first, for its availability in our local animals milk, second as mentioned by Sevec *et al*., (15) the dominant end product is L-lactic acid and third, for the few studies in Iraq concerning with it.

Kalhotka *et al*., (16) isolated *Enterococcus faecium, E. mundtii, E. durans* from9 samples of goat milk and cheeses. In the study of Edalatian *et al*., (17) traditional Iranian cheeses (Lighvan and Koozeh) made of raw ewe's milk or mixtures of ewe's and goat's milk has been studied, and *Enterococcus faecium* and *Enterococcus faecalis* were found to be dominant among the isolates in all batches.

Eleven strains *Enterococcus faecium* have been isolated from cows and sheep milk (18).

The strong positive growth at 10°C, negative growth at 45°C, negative reactions of pyrrolidonylarylamidase (PYR) and hippurate varible may be suggested to the new local Iraqi strain.

In respect of crude extract, HPLC analysis for the dried 5ml of whey medium has shown that *Enterococcus faecium* (B4) has a higher concentration of L- lactic acid with about (0.659g/l) while the D- lactic was about (0.174g/l).

Concerning the purified extract, B4 has been selected due to its high L- lactic concentration and the treatment with $NaHCO_3$ has shown bubbles of CO_2 in part 2 and 3 as a result of the chemical reaction with the total lactic acid, whereas part 1 may contain proteins and other metabolites.

By using HPLC, a sharp peak has represented L-lactic acid with a purity percent of 72.3% , and 38.2% for D-lactic acid with no visible peak for D-lactic may be due to small area of 16713.

Cao *et al*., (12) elevated that Amberlite resin IRA-400 was successfully applied for the separation of L-(+)-lactic acid from fermentation broth at pH above and below the pK_a (3.86) and found that 1.0 M H_2SO_4 could be used for the elution of lactic acid at pH 5.0 with high recovery with yield of 86.21% and the maximum adsorption capacity (197.09 mg/g wet resin) at pH 5.0 was much higher than that at pH 2.0 (106 mg/g wet resin), but the adsorption performance of resin for lactic acid at pH 2.0 was not affected

by salts ions in fermentation broth and inorganic acids used for adjustment of pH in which the total yield was 92.11% when water was used as eluent.

Also, a weak anion exchanger Amberlite IRA-92 was used (13), and it was found that the yield, purity and productivity about 82.6%, 96.2% and 1.16 g LA/(g-resin day), respectively after optimization.

The deproteinized whey was an attractive medium for the production of lactic acid by free and coimmobilized *Lactobacillus casei* and *Lactococcus lactis* cells using fedbatch culture (19).

In conclusion, Iraqi raw animal milk has a high variety of bacterial strains, which could be isolated and implemented in different industries. *Enterococcus faecium* is a good species that has the ability to produce lactic acid especially L- type as dominant product.

إنتاج حامض اللبنيك بوساطة العزلة العراقية المحلية *Enterococcus faecium*

ميثم ايوب الحمداني* آمال كاظم غضبان الاسدي** ووصال عودة حسن الحلفي***

* قسم علوم الحياة،كلية التربية للعلوم الصرفة، جامعة البصرة،

** قسم التقانات الاحيائية، كلية الزراعة, جامعة البصرة ،

***قسم علوم الحياة، كلية العلوم، جامعة البصرة.

الخلاصة

يعد حامض اللاكتيك حامضا كاربوكسيليا مهما و يستخدم في جوانب الحياة المختلفة. تضمنت الدراسة الحالية الحصول على عزلة نقية من بكتريا حامض اللبنيك متجانسة التخمر وهي *Enterococcus faecium* وتشخيصها بالفحوصات المظهرية مع اختبارات فسيولوجية وكيموحيوية.

وقد اظهرت نتائج الدراسة قابلية العزلة على انتاج حامض اللبنيك نوع L بتركيز مرتفع وتركيز منخفض من حامض اللبنيك نوع D مقارنة بالسيطرة.

استخلص حامض اللبنيك وتمت تنقيته جزئيا بواسطة عمودالتبادل الايوني الراتنج امبرلايت IRA400 و اختبرت الاجزاء المستخلصة المستحصل عليها بوساطة اختبار كشف الاحماض الكاربوكسيلية بالاضافة الى تحليلات جهاز كروماتوغرافيا السائل عالي الكفاءة HPLC، والذي اظهر ان تركيز حامض اللبنيك نوع L كان 0.659 غم/لتربينما كان تركيز نوع D 0.174 غم/لترفي الاجزاء الخام مع ظهور قمتين واحدة بمساحة 322854 لنوع L

بينما لم تظهر قمة مرئية بالنسبة لنوع D بسبب كونها ذات مساحة صغيرة 72214 في الشكل، في حين ان القمة الثانية ذات المساحة 203238 ربما تعود للتداخل بين النوعين D و L من حامض اللبنيك. وقد وصلت الاجزاء المنقاة الى نسبة نقاوة مقدارها 72.3% بالنسبة للنوع L و 38.2% بالنسبة للنوع D وايضا مع قمة حادة واحدة للنوع L بمساحة 43639 وعدم وجود قمة مرئية لحامض اللبنيك نوع D لصغر مساحتها البالغة 16713 .

REFERENCES

1. Groot, W.; Van Krieken, J.; Sliekersl, O. and De Vos, S. (2010). Production and purification of lactic acid and lactide. Cited by: Auras, R.; Lim, L.- T.; Selke, S. E. M. and Tsuji, H. (Eds). Poly(lactic acid): Synthesis, structures, properties, processing, and applications. John Wiley & Sons, Inc., Hoboken, New Jersey, 499pp.
2. Jem, K. J.; Van der Pol, J. F. and De Vos, S.(2010). Microbial lactic acid, its polymer Poly(lactic acid), and their industrial applications. Cited by : Chen, G.- Q. (Ed). Plastics from bacteria, natural functions and applications.Volume 14, Springer-Verlag Berlin Heidelberg, 450pp.
3. Prescott, L. M.; Harley, J. P. and Klein, D. A. (Eds). (2002). Microbiology, Fifth Edition, The McGraw−Hill Companies, 1147pp.
4. Lauková, A. and Kuncová, M. (1991). Lactic acid production and urease activity in strains of *Enterococcus faecium* found in the rumen and their genetic stability. *Veterinární medicína(Phara)*, 36(6):335- 340 (Abstract).
5. Nolasco-Hipolito, C.; Zarrabal, O. C.; Kamaldin, R. M.; Teck-Yee, L.; Lihan, S.; Bujang, K. B. and Nitta, Y. (2012). Lactic acid production by *Enteroccocus faecium* in liquefied sago starch. AMB Express, 2:53: 1- 9.
6. Bulut, C. (2003). Isolation and molecular characterization of lactic acid bacteria from Cheese. MSc. Thesis, İzmir Institute of Technology İzmir, Turkey, 102pp.
7. Grainger, J.; Hurst, J. and Burdass, D. (2001). Basic practical microbiology, A manual. The Society For General Microbiology, UK. 27pp.
8. Harisha, S. (Ed). (2007). Biotechnology procedures and experiments handbook. Infinity Science Press LLC, New Delhi, India, 694pp.
9. Harrigan, W. F. and McCans, M. E., (1976). Laboratory Methods in Food & Dairy Microbiology. Academic press, London. Cited by: (Al- Asady, 2003).
10. Al- Asady, A. K. G. (2003). Improvement of dextran production from a local isolate of *Leuconostoc mesenteriodes* . PhD.Thesis, College of agriculture, University of Basrah, 145pp.
11. Al- Chezzy, K. W. M. (2003). Identification of local isolate of *Lactobacillus delbrueckii* subsp. *bulgaricus* and using it to production lactic acid from local renewable resources. PhD.Thesis, College of agriculture, University of Basrah, 138pp.

12. Cao, X.; Yun, H. S. and Koo, Y.- M. (2002). Recovery of L-(+)-lactic acid by anion exchange resin Amberlite IRA-400. *Biochemical Engineering Journal*, 11 (2–3):189-196.
13. Tong, W.- Y.; Fu, X.- Y.; Lee, S.- M.; Yu, J.; Liu, J.- W.; Wei, D.- Z. and Koo, Y.- M. (2004). Purification of L (+)-lactic acid from fermentation broth with paper sludge as a cellulosic feedstock using weak anion exchanger Amberlite IRA-92. *Biochemical Engineering Journal*, 18 (2): 89- 96.
14. Shriner, R. L.; Hermann, C. K. F.; Morrill, T. C.; Curtin, D. Y. and Fuson, R. C. (Eds). (2004). The Systematic identification of organic compounds. Chapter 9. Chemical tests for functional groups. Eigth edition, John Wiley & Sons. Inc, 723pp.
15. Švec, P. and Devriese, L. A. (2009). Genus I. *Enterococcus*. Cited by: (De Vos, P.; Garrity, G. M.; Jones, D.; Krieg, N. R.; Ludwig, W.; Rainey, F. A.; Schleifer, K.- H. and Whitman, W. B. (Eds). Bergey's Manual of Systematic Bacteriology, Second edition, Volume three, Springer, New York, 1422pp.
16. Kalhotka, L.; Manga, I.; Přichystalová, J.; Hůlová, M.; Vyletělová, M. and Šustová, K. (2012). Decarboxylase activity test of the genus Enterococcus isolated from goat milk and cheese. *Acta Veterinaria BRNO*, 81: 145–151.
17. Edalatian, M. R.; Najafi, M. B. H.; Mortazavi, S. A.; Alegría, A.; Nassiri, M. R.; Bassami, M. R. and Mayo, B. (2012). Microbial diversity of the traditional Iranian cheeses Lighvan and Koozeh, as revealed by polyphasic culturing and culture-independent approaches. *Dairy Science and Technology*, 92:75–90.
18. Kacem, M.; Zadi-Karam, H. and Karam, N.- E. (2003). Identification of lactic acid bacteria isolated from milk and fermented olive oil in western Algeria. Actes Inst. Agron. Vet. (Maroc), 23(2-4): 135-141.
19. Roukas, T. and Kotzekidou, P. (1998). Lactic acid production from deproteinized whey by mixed cultures of free and coimmobilized *Lactobacillus casei* and *Lactococcus lactis* cells using fedbatch culture. *Enzyme and Microbial Technology*, 22:199-204.