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Comparative study on lactic acid production of different lactic acid bacteria through RP-HPLC method

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Abstract

In this study, a simple and reliable HPLC procedure has been developed for determination of lactic acid (LA) in liquid bacterial cultures. The lactic acid concentration is used as a criterion for strain selection. Eight LAB strains have been isolated from silage inoculants and food grade rice seeds. All strains showed the potential to produce more than 0.1 g/L LA, three strains produced LA above 0.8g/L, and one strain has the potential to produce 2.94g/L LA.

Keywords biogas, bio-resources, fallen leaves, methane, *Tectona grandis*

Introduction

The increasing Lactic acid bacteria (LAB) are ubiquitous microorganisms that are known in relation to the storage of food products. They are used in the fermentation of food, contributing to the taste and texture of the fermented products [1-3]. LAB are classified as homo-fermentative and hetero-fermentative, producing L (+) lactic acid or D (-) lactic acid, or racemic mixture of both the isomers. The genera *Lactococcus, Lactobacillus, Streptococcus, Pediococcus, Aerococcus, Carnobacterium, Enterococcus, Oenococcus, Tetragenococcus, Vagococcus* and *Weisella* are the main representatives of LAB [4-7]. Their main use is in the form of probiotics in humans and animals [8-11] and these are successfully used for plant diseases control, often in combination with other beneficial microorganisms, or for accelerating the decomposition of organic matter in the soil [12].

Lactic acid bacteria reveals a favorable effect on inhibiting the growth of pathogenic bacteria such as *E.coli, Salmonella spp., Listeria spp*. [13–16]. They synthesize inhibitory substances such as organic acids, hydrogen peroxide, acetoin, butanediol, acetaldehyde, benzoate, bacteriolytic enzymes, bacteriocins [17-19]. Most often, the lactic acid bacteria exhibit an antimicrobial effect on bacterial pathogens by the production of metabolites such as lactic and acetic acid, and subsequently decreasing the pH [20]. The low pH is the reason for increasing the solubility of organic acids in lipids, allowing them to break through the cell membrane and reach in to the cytoplasm of pathogens [21].

The levels and type of organic acids produced during the fermentation process depend on the species of microorganisms and growth conditions [22].

Productivity is an important factor in assessing the potential of a microbial strain [23]. The potential for LA production varies between the different LAB species, as well as between the individual strains within a species. Therefore, a simple and reliable analytical method is required for the routine laboratory microbial strain selection. It is also interesting to select the cheapest media that would allow the highest lactic acid concentration to be obtained.
The aim of the study was to determine the invitro production of certain organic acids (lactic acid) in MRS broth by LAB cultured in cheap glucose medium. The used HPLC method is easy and quick and allows a reliable measurement of LA in wide range of concentrations.

Methodology

Bacterial isolation and determination

Sources for isolation of Lab

The different Lactic Acid Bacteria were isolated from various sources.
I) two commercial silage inoculants;
II) food grade rice seeds (end-user packed products)
The silage samples were taken from local dairy farms and the food grade packed rice samples were bought from the local retail stores.

Culture media and bacteria isolation

The samples were analyzed for total solids (TS), volatile solids (VS) and pH by standard methods [13], and Lactobacillus MRS Agar and Lactobacillus MRS Broth were purchased from HiMedia Laboratories Pvt. Ltd (India).
The bacteria were isolated through enrichment in MRS broth. About 0.1 g of each silage inoculant and 10 g of the rice samples were blended with 100 ml deionized water, and shaken manually for 5 minutes. Ten ml aliquot of the blended samples was inoculated into 100 ml MRS broth in 250 ml Erlenmeyer flask. The flasks were kept static at 37 °C for 7 days. After 7 days, the cultures from the different sources were used to make diluted solution up to 10-6.
Then, 0.1 ml of the final dilution (10-6) was applied on MRS agar plate and the plates were kept at 37 °C for 3 days.
Individual colonies from each isolate were chosen at random and sub-cultured in MRS broth for 3 days at 37 °C to propagate the growth of the individual isolates.

Production and extraction of lactic acid

Initially, 0.5 ml of the MRS broth with each LAB isolate was added to 9.5 ml of 5% sterile glucose solution in a test tube. The inoculated glucose solutions were kept at 37 °C for 96h. One ml of the solutions was taken regularly at 24h interval for determination of LA quantity. Lactic acid extraction was performed according to method [24] with minor modifications.

Bacteria identification

The isolated bacterial strains were determined using the identification system Biolog's AN Phenotype MicroArray for microbial cells (Biolog, Inc., USA). The principle of the test was based on the phenotypic detection of the biochemical characteristics of the examined microorganism.
Eight different Lab species were isolated and used as biological agents in the study:
Pediococcus acidilactici
Lactobacillus plantarum
Lactobacillus fermentum
Pediococcus acidilactici
Lactobacillus buchneri
Lactobacillus delbrueckii, ss lactis
Lactobacillus paracasei, ss paracasei

Lactobacillus lactis

Analytical quantification of lactic acid
The identification and quantification of the LA produced by the isolates were performed using the description of the RP-HPLC method developed by us. Lactic acid was identified by comparison of retention times and area values obtained by injecting a standard of lactic acid.

Chemicals
Sulfuric acid (HPLC-grade) was bought from Sigma Aldrich (Germany), Na2SO4 (analytical grade) was purchased from Valerus (Bulgaria) and Lactic Acid standard (CRM) was purchased from AlfaPharm (Bulgaria), while 5% sterile glucose solution was bought from Actavis (Bulgaria).

HPLC equipment
A RP-HPLC apparatus (Ultimate 3000, Varian), equipped with a UV-VIS detector, LPG - 3400 A pump, and auto sampler WPS - 3000 SL, was used..

Column and mobile phase
A RP column C18 (250, 4.6 mm, 5 mm in particle size), equipped with the precolumn was a matter of choice for the study. The chromatographic conditions were set to provide isocratic flow of 1 ml/min and injection volume of 100 µl; 0.1M Na2SO4 was used as mobile phase, pH=2.65, acidified with conc. H2SO4; UV detection was monitored at 210 nm..

Software
The data processing was carried out automatically by the software of the HPLC system (Chromeleon 6.80 SR1, Dionex). The mean value and standard error were calculated from the data obtained from six replicates of each treatment. All statistics were performed, using descriptive statistics module in Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA)..

Standards and Calibration
Mobile phase and a certified reference material (CRM) for Lactic Acid with concentration of 10 mg/ml were used for preparation of calibration solutions with concentration – 10 µg/ml, 50 µg/ml, 150 µg/ml, 500 µg/ml, 1500 µg/ml, 3000 µg/ml..

Sample preparation
One ml of each sample's glucose solutions was centrifuged at 4000 rpm for 10 minutes; the aliquot was filtered through 0.22 µm syringe filter and transferred to chromatographic vials for analysis..

Method validation
System suitability
The suitability of the system was evaluated by the values of the percent coefficient variation (%CV) and the retention time of the LA from six replicates of calibration solution with concentration of 150 µg/ml. The criterion for acceptance of both indices was ± 2%.

Limit of detection (LOD) and limit of quantification (LOQ)
The LOD and LOQ were calculated on the basis of the slope and the intercept of the calibration curve.

Linearity
The linearity of the analytical procedure was checked by injecting 6 calibration solutions and by plotting the acquired signals (chromatographic peak area) against the corresponding concentrations. From that regression equation and the regression coefficient were calculated.

Accuracy and precision

Both characteristics were determined by calculating the variability of six replicates of matrix sample (0.5 ml pure MRS broth in 9.5 ml 3% sterile glucose solution) spiked with LA to final concentration of 150 µg/ml, during the single day of the examination and between the examinations on different days.

Robustness

In the present study, a variation of pH (±0.2) of the mobile phase was used to demonstrate that the method remains unaffected by different variations of the method parameters.

Stability

The stability of the calibration solutions of LA was determined by storing the vials in auto sampler for 96 hrs and then, analyzed for second time.

Results and Discussion

Validation of characteristics of the method

The retention time (Rt) of LA for the settings of the HPLC system was 5.761 min and the peak shape had 10% asymmetry of 1.46. LOD and LOQ were found to be 1 µg/ml and 6 µg/ml. The coefficient of linearity regression (R2) was 0.9996. The recovery of analyte (accuracy) was 99.684%. The coefficient of variation for repeatability and intermediate precision were 0.539 and 0.542, respectively. A summary of the method characteristics is given in Table 1

Table 1 Characteristics of the method validation

Characteristic	Result	Criteria
Specificity	5.761±0.021; no interference at Rt	No interference at Rt
Linearity	10µg/ml-3000 µg/ml	-
Coefficient of regression (R2)	0.9996	>0.99
Repeatability (%CV)	0.539	<1.00
Intermediate precision (%CV)	0.542	<2.00
Accuracy (%Mean ± SD)	99.684 ± 0.891	90-110
LOD (µg/ml)	1.0	S/n=3:1
LOQ (µg/ml)	6.0	S/n=10:1
Robustness (%RSD)	0.98	<2%

Lactic acid production by Lab in MRS broth

The Chemical Oxygen Demand (COD) is used to quantify the amount of organic matter in waste streams All the isolated strains started to produce LA shortly after inoculation in the glucose solution. After one hour of the experiment, the measured quantity of LA ranged between 10.297µg/ml and 45.553 µg/ml. The levels increased dynamically and at 96th hour, the range produced by the different strains varied between 135.914 µg/ml and 2946.132 µg/ml.

At the end of the experiment *Lactobacillus* plantarum produced the highest levels of LA, followed by Lactobacillus delbrueckii, ss lactis. *Lactobacillus buchneri* produced the lowest level of LA.

A summary of the production of LA by the selected bacterial isolates in dynamics is given in Table 2

Table 2 Summary of the production of LA by the selected bacterial isolates in dynamics

Time, hours	1 h	24 h	48 h	72 h	96 h
Bacterial isolates:	**(µg/ml)***	**(µg/ml)***	**(µg/ml)***	**(µg/ml)***	**(µg/ml)***
Pediococcus acidilactici	**25.110 ± 0.046**	**335.881 ± 0.018**	**716.553 ± 0.509**	**799.537 ± 0.371**	**995.107 ± 0.640**
Lactobacillus plantarum	**31.259 ± 0.046**	**417.599 ± 0.400**	**890.987 ± 0.545**	**2366.689 ± 0.411**	**2946.132 ± 0.495**
Lactobacillus fermentum	**4.555 ± 0.034**	**67.04 ± 0.462**	**81.263 ± 0.755**	**174.867 ± 0.425**	**215.96 ± 0.522**
Pediococcus acidilactici	**17.721 ± 0.054**	**266.031 ± 0.539**	**395.621 ± 0.164**	**653.368 ± 0.612**	**812.944 ± 0.283**
Lactobacillus buchneri	**2.252 ± 0.046**	**32.71 ± 0.393**	**39.652 ± 0.38**	**110.261 ± 0.41**	**135.914 ± 0.744**
Lactobacillus delbrueski, ss lactis	**27.957 ± 0.054**	**374.355 ± 0.426**	**796.471 ± 0.543**	**1432.249 ± 0.373**	**1783.828 ± 0.416**
Lactobacillus paracasei, ss paracasei	**10.297 ± 0.037**	**154.421 ± 0.334**	**188.638 ± 0.289**	**398.786 ± 0.374**	**496.261 ± 0.609**
Lactobacillus lactis	**2.936 ± 0.035**	**42.068 ± 0.298**	**51.128 ± 0.359**	**143.4 ± 0.274**	**178.898 ± 0.409**

The results are expressed as average of six replications and standard error of the mean

According to the COD estimation, our study showed that the fallen teak leaves biomass is a potentially The method's characteristics comply with the acceptance criteria. The LOD corresponded to the level described by Kuo et al. [23], even though the settings of the HPLC system were slightly different.

Hor and Liong [25] found that the concentrations of the lactic acid produced by LAB species were strain-dependent and all strains showed higher concentration of lactic acid than acetic acid.
The examined eight different species produced different quantities of LA.
In our study, Lactobacillus plantarum demonstrated the highest production of LA that corresponds with the study of Georgieva et al. [26], in which the strain of Lactobacillus plantarum produced 13.68 g/L LA in MRS broth in 2% glucose medium.
Lactobacillus delbrueski, ss lactis produced 17.88g/L LA that was lower in comparison with the findings of Nakano et al. [27]. The author used three different neutralizing agents - Ca(OH)2 , NH4OH, and NaOH – in a simultaneous saccharification and fermentation process that stimulate the production of LA. On the other hand, they explained that high glucose concentrations in the growth medium might lead to the inhibition of bacterial growth (glucose repression). That might be a plausible explanation for why certain strains in the current study produces relatively low concentration of LA like - *Lactobacillus fermentum* 215.96g/L, *Lactobacillus lactis* 0.179g/L, *Lactobacillus buchneri* 0.135g/L, so further investigation is needed to determine the actual number of bacterial cells in the glucose solution in relation to LA concentration.
Te'llez-Luis et al. [28] reported that 9% glucose concentration is suitable for production of LA by *Lactobacillus delbrueckii*, however the team used a Mercier medium rich in yeast extract and mineral salts. Hofvendahl and Hahn-Hagerdal [30] stated that lactic acid bacteria have limited capacity to synthesize B-vitamins and amino acids and this can be overcome by supplementation of yeast extracts rich in B-vitamins and amino acids. Kuo et al. [23], used higher initial glucose content (between 50g/L and 220g/L) plus yeast extract, and observed higher production of LA in *Lactobacillus paracasei*, *Lactobacillus fermentum* and *Lactobacillus plantarum* as compared to the levels determined in our study. The relatively low levels of LA, obtained in our study, might be explained with the limiting effect of nitrogen and vitamins, because no nitrogen or vitamin source was added in the fermentation media in our experiment.
The lowest level of LA in the study was estimated in the Lactobacillus buchneri strain. That might be explained with the fact that this species has a wider range of metabolic activities than some of its faster growing relatives in the family of LAB. It has the ability to anaerobically convert lactic acid to acetic acid and 1,2-propanediol in microbiological media [30]. Zhang et al. [31] reported that *Lactobacillus buchneri* produced mainly 1,2-propanediol during growth in mMRS..

Conclusion

The proposed method is easy and reliable for fast quantification of LA in glucose culture media. The highest production of 2.946 g/l of LA was observed in Lactobacillus plantarum strain, while the lowest production of LA was observed in Lactobacillus buchneri strain. The levels of nitrogen and vitamins were observed to be limiting factors for the bacterial growth.

References

[1] C. Nordqvist **(2004)**. Lactic acid bacteria - their uses in food. Medical News Today. Retrieved from http:// www.medicalnewstoday.com/releases/14023.php
[2] F. Leroy, L. De Vuyst **(2004)**. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends Food Sci. Technol., **15:** 67-78.
[3] S. A. Abdullah, M. M. Osman **(2010)**. Isolation and Identification of Lactic Acid Bacteria from Raw Cow Milk, White Cheese and Rob in Sudan. Pakistan Journal of Nutrition, **9:**1203-1206.
[4] L. Axelsson **(2004)**. Lactic acid bacteria: classification and physiology. In: S. Salminen, A. von Wright, A. Ouwehand, ed. Lactic acid bacteria: microbiological and functional aspects. Marcel Dekker Inc, New York, pp. 1-66.
[5] D. Ercolini, G. Moschetti, G. Blaiotta, S. Coppola **(2001)**. Behavior of variable V3 region from 16S rDNA of lactic acid bacteria in denaturing gradient gel electrophoresis. Curr. Microbiol., **42:** 199-202.
[6] J. M. Jay **(2000)**. Fermentation and fermented dairy products. Modern food microbiology. 6th edition. Gaithersburg, USA: An Aspen Publication, Aspen Publishers, Inc., pp. 113-130.
[7] W. H. Holzapfel, P. Haberer, R. Geisen, J. Björkroth, U. Schillinger **(2001)**. Taxonomy and important features of probiotic microorganisms in food nutrition. Am. J. Clin. Nutr., **73:** 65-73.
[8] A. Sofyan, M. Angwar, H. Herdian, E. Damayanti, L. Istiqomah, A. Febrisiantosa, H. Julendra et al. **(2012)**. Performance Enhancement and Immunity Profile of Broiler Treated Feed Additive Containing Lactic Acid Bacteria and Ganoderma lucidum. Med. Pet., **35:** 201-206.
[9] M. A. K. Torshizi, S. H. Rahimi, N. Mogjani, S. Esmaeilkhanian, J. L. Grimes **(2008)**. Screening of indigenous strains of lactic acid bacteria for development of a probiotic for poultry. Asian-Australian J. Anim. Sci., **21:** 1495-1500
[10] K. Angmo, A. Kumari, Savitri, T. C. Bhalla (2016). Probiotic characterization of lactic acid bacteria isolated from fermented foods and beverage of Ladakh. LWT - Food Sci.Technol., 66: 428-435.
[11] J. L. Ellis, A. Bannink, I.K. Hindrichsen, R. D. Kinley, W. F. Pellikaan, N. Milora, J. Dijkstra **(2016)**. The effect of lactic acid bacteria included as a probiotic or silage inoculant on in vitro rumen digestibility, total gas and methane production. Anim. Feed Sci. Tech., **211:** 61-74.
[12] N. Limanska, T. Ivanytsia, O. Basiul, K. Krylova, V. Biscolla, J. Chobert, V. Ivanytsia et al. **(2013)**. Effect of Lactobacillus plantarum on germination and growth of tomato seedlings. Acta Physiol. Plant., **35:** 1587-1595.
[13] L. Istiqomah, S. N. Hayati, E. Damayanti, H. Julendra, A. A. Sakti, T. Untari **(2013)**. Performance and Meat Quality of Broilers Infected with Escherichia coli and Administered with Bio Additive, Probiotic, and Antibiotic. Med. Pet., **36:** 14-20,
[14] M. Nouri, F. Rahbarizadeh, D. Akhmadvand, F. Moosakhani, E. Sadeqzadeh, S. Lavasani, V. K. Vishteh **(2010)**. Inhibitory effects of Lactobacilus salivarius and Lactobacillus crispatus isolated from chicken gastrointestinal tract on Salmonella enteritidis and Escherichia coli growth. Iranian J. Biotechnol., **8:** 32-37.
[15] S. N. Jannah, A. Dinoto, K. G. Wiryawan, I. Rusmana **(2014)**. Characteristics of Lactic Acid Bacteria Isolated from Gastrointestinal Tract of Cemani Chicken and Their Potential Use as Probiotics. J. Anim. Sci. Technol., **37:** 182-189.
[16] F. Manini, M.C. Casiraghi, K. Poutanen, M. Brasca, D. Erba, C. Plumed-Ferrer **(2016)**. Characterization of lactic acid bacteria isolated from wheat bran sourdough. LWT - Food Sci. Technol., **66:** 275-283.
[17] T. C. Chung, L. Axelsson, S..E. Lindgren, W. J. Dobrogosz **(1989)**. In vitro studies on reuterin synthesis by Lactobacillus reuteri. Microbial Ecol. Health Dis., **2:** 137-144.

[18] P. A. Vanderbergh **(1993)**. Lactic acid bacteria, their metabolic products and interference with microbial growth. FEMS Microbiol. Rev., **12:** 221-237.
[19] L. De Vuyst, E. J.Vandamme **(1994)**. Antimicrobial potential of lactic acid bacteria. In. Bacteriocins of lactic acid bacteria. 1st ed. Blackie Academic and Professional, Chapman and Hall, Glasgow, pp 91-142.
[20] A. S. Naidu, W. R. Bidlack, R. A. Clemens **(1999)**. Probiotic Spectra of Lactic Acid Bacteria (LAB). Crit. Rev. Food Sci. Nutr., **39:** 113-126.
[21] F. Djadouni, M. Kihal **(2012)**. Antimicrobial activity of lactic acid bacteria and then spectrum of their biopeptides against spoiling germs in foods. Braz. Arch. Biol. Techn., **55:** 435-443.
[22] S. Lindgren, W.J Dobrogosz (1990). Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiol. Rev., **87:**149-164
[23] Y. Kuo, Shuo-Fu Yuan, Chun-An Wang, Yin-Jung Huang, Gia-Luen Guo, Wen-Song Hwang **(2015)**. Production of optically pure L -lactic acid from lignocellulosic hydrolysate by using a newly isolated and D -lactate dehydrogenase gene-deficient Lactobacillus paracasei strain. Bioresource Technol., **198:** 651-657.
[24] D. Asami, Yun-Jeong Hong, Diane M. Barrett, Alyson E. Mitchell **(2003)**. Comparison of the Total Phenolic and Ascorbic Acid Content of Freeze-Dried and Air-Dried Marionberry, Strawberry, and Corn Grown Using Conventional, Organic, and Sustainable Agricultural Practices. J. Agric. Food Chem., **51:**1237-1241.
[25] Y. Hor, Min Tze Liong **(2014)**.Use of extracellular extracts of lactic acid bacteria and bifidobacteria for the inhibition of dermatological pathogen Staphylococcus aureus. Dermatologica Sinica, **32:** 141-147.
[26] R. Georgieva, P. Koleva, D. Nikolova, D. Yankov, S. Danova **(2009)**. Growth parameters of probiotic strain Lactobacillus plantarum, isolated from traditional white cheese. Biotechnol. & Biotechnol. Eq., 23: 862-865
[27] S. Nakano, Ch. U. Ugwu and Y. Tokiwa **(2012)**. Efficient production of D -(-)-lactic acid from broken rice by Lactobacillus delbrueckii using Ca(OH) 2 as a neutralizing agent. Bioresour. Technol., **104:** 791–794.
[28] S. Te'llez-Luis, A. B. Moldes, M. Va´zquez and J. L. Alonso **(2003)**. Alternative media for lactic acid production by Lactobacillus delbrueckii NRRL B-445. Food Bioprod. Process, **81:** 250-256.
[29] K. Hofvendahl and B. Hahn-Hagerdal **(2000)**. Factors affecting the fermentative lactic acid production from renewable resources. Enzyme Microb Technol., **26:** 87-107.
[30] O. Elferink, S. J. W. H., J. Krooneman, J. C. Gottschal, S. F. Spoelstra, F. Faber, F. Driehuis (2001). Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by Lactobacillus buchneri. Appl. Environ. Microbiol., **67:** 125-132.
[31] C. Zhang, M. J. Brandt, C. Schwab, M. G. Gänzle **(2010)**. Propionic acid production by cofermentation of Lactobacillus buchneri and Lactobacillus diolivorans in sourdough. Food Microbiol., **27:** 390-395.