International Food Research Journal 23(6): 2714-2718 (December 2016)
Journal homepage: *http://www.ifrj.upm.edu.my*

Enhancement of lactic acid bacteria growth using sheep hooves hydrolyzate

[1*]Ahmed-Gaïd, K., [1]Boukhemis, M. and [2]Djeghri-Hocine, B.

[1]*Laboratory of Applied Biochemistry and Microbiology, Department of Biochemistry, Faculty of Sciences, Badji Mokhtar University BP12, 23000 Annaba, Algeria*
[2]*Nationale High Ecole of Marine Science and Coastal Development, Algiers, Algeria*

Article history

Received: 27 September 2015
Received in revised form: 21 January 2016
Accepted: 15 February 2016

Keywords

Sheep hooves
Enzymatic hydrolysis
Growth medium
Lactic acid bacteria, MRS

Abstract

Sheep hooves were subjected to an enzymatic hydrolysis using papain to produce hooves hydrolyzate (HH). Some HH based formulations were tested to support *Lactobacillus farciminis* DSM20184 growth, as a result, the combination of HH and 20 g L^{-1} of glucose was selected as Hooves Medium (HM) and compared with MRS to cultivate other lactic acid bacteria. Generally, HM showed a good performance to support the strains growth, *L. fermentum* DSM20049 and *L. farciminis* showed the most significant results with maximal OD600 that are respectively 67.24% and 33.77% higher than those obtained on the standard medium MRS. Hence, the formulation of HM could constitute a good way to exploit sheep hooves and may be proposed as a suitable solution to reduce LAB biomass production costs.

Introduction

Recently, formulation and optimization of new growth media became an important research field. This is due to the high costs of their components, and the need to enhance biomass and metabolites productions for technological aims. In fact, media high costs are essentially related to the nitrogen sources, such as peptones derived mainly from casein, soya and meat. (Vazquez and Murado 2008). To substitute the costly ingredients and to recover microorganisms requirements, several studies reported the formulation of media using inexpensive substrates like vegetables: potato extracts (Gaudreau *et al*., 2002), dates (Nancib *et al*., 2005), molasses (Coelho *et al.*, 2011), corn stover (Cui *et al*., 2011), tomato pomace (Belmessikh *et al*., 2013), split pea and dry figs (Kassas *et al*., 2015). Other studies seemed to be more interesting reporting the use of cheaper substrates, which are biological wastes, for example: fish wastes (Clausen *et al*., 1985; Horn *et al*., 2005; Aspmo *et al.*, 2005; Vazquez and Murado, 2008), sheep horns (Kurbanoglu, 2004) and tuna heads (Safari *et al*., 2009).

To evaluate the efficiency of new formulated media, those should be tested to cultivate fastidious microorganisms, that is why, Lactic Acid Bacteria (LAB) are the most group of microorganisms used for this aim. In spite of the availability of important results reporting the formulation of inexpensive media for biomass and metabolites productions from LAB, (Horn *et al.*, 2005; Vazquez and Murado, 2008), MRS medium (de Mane Rogosa and Sharpe) stilled the most used laboratory medium, however, this medium showed a major inconvenient looking to its high cost. Nowadays, LAB biomass production become more and more important because of the characterization of new strains with beneficial properties which are exploited in food technology (Georgiva *et al.*, 2009), in cosmetic and in pharmaceutical industry (Franz *et al*., 2010; LeBlanc *et al*., 2010), hence, it is important to find a solution to reduce their biomass production costs.

The present study aims to exploit sheep hooves to formulate a growth medium for LAB. This substrate is not exploited in any field in Algeria, moreover, it constitutes a source of environment pollution and health diseases, because, as other animal by-products, it is usually buried or incinerated with no pretreatment. This work describes the use of sheep hooves hydrolyzate as a nitrogen source and its combination with other components to formulate a cheap growth medium for five LAB strains from different origins. A comparative study with MRS medium was carried out to evaluate the performance of HM as growth medium.

Materials and Methods

Raw material

Sheep hooves were collected from the slaughterhouse immediately after the slaughtering.

*Corresponding author.
Email: *agkelthoum@gmail.com*
Tel: +213 797 17 81 87; Fax: +213 38 87 26 53

They were washed carefully in running water, dried at 50°C for 8h and were cut into small pieces; those were ground to obtain granular powder of 2-3 mm of size and frozen in boxes at -20°C until use.

Enzymatic hydrolysis

Hooves powder was mixed with distilled water (1:2 w/v) and the pH was adjusted to 6 by adding 1N HCl. The enzymatic hydrolysis using papain at 0.5% (w/w): enzyme to substrate ratio. The mixture was dispensed into 250-ml Erlenmeyer flasks; those were placed in a rotary shaker for 24h at 65°C and 150 rpm. The hydrolysis was terminated by the inactivation of the enzyme at 90°C for 20 min. The hydrolyzate was centrifuged 4000 rpm for 25 min and filtered to eliminate the fat layer, then the supernatant was recuperated (Hooves Hydrolyzate: HH).

Microorganisms

Five strains of lactic acid bacteria were used in this study: *Lactobacillus fermentum* DSM 20049 and *Lactobacillus farciminis* DSM 20184 from the culture collection DSMZ. *Lactobacillus plantarum* BH14 and *Lactobacillus brevis* CHTD27 from the collection of Laboratoire de Biologie des Microorganismes et Biotechnologie (LBMB) (Essenia, Oran, Algeria) were originally isolated from raw camel milk collected in Tindouf (Roudj *et al.*, 2009), and *Lactobacillus rhamnosus* sp. isolated from baby feces. The strains were stored as frozen stock held at -20°C in milk. Inoculums were prepared by adding a colony to MRS broth and incubating it at 37°C for 18 h, before inoculation OD_{600} of the inoculums were adjusted to 0.6.

Growth media and bacterial growth estimation

Media were composed of HH, 20 g L^{-1} of glucose and 5 g L^{-1} of yeast extract as following: HH: composed only of HH, HH-G: HH+ glucose, HH-G-Y: HH+ glucose +yeast extract, Y-G: yeast extract + glucose. MRS medium was used as standard for media comparison. pH of the media was adjusted to 6.5 by adding 0.1N NaOH . After autoclaving at 121°C for 15 min, media were cooled and inoculated with 3% of the inoculum and incubated at 37°C for 24 h. After fermentation, biomass concentrations were estimated by measuring optical densities of the samples at 600 nm (OD_{600}). Samples were diluted with their corresponding media to ensure that the OD_{600} value is less than 0.6.

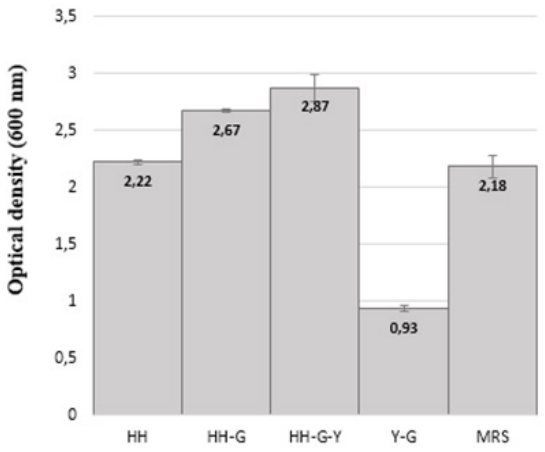
Growth kinetics

Growth kinetics of the five LAB strains on HM and MRS were studied for 28 h at 37°C, the curves were monitored by measuring OD_{600} every 2 hours. Analysis of variance of the results using MINITAB software version 16.2.1.0 was carried out to analyze the significance of the differences between the growth on MRS and HM ($p < 0.05$).

Results and Discussion

Selection of hooves medium composition

Growth results of *Lactobacillus farciminis* DSM20184 on the four HH media and the MRS medium are represented in figure 1. Those were significantly different ($p<0.05$), with finals OD600 varying between 0.93 and 2.87. The best growth results were those of HH-G and HH-G-Y showing OD_{600} of 2.67 and 2.87 respectively, which are significantly higher than that obtained on the standard medium MRS. HH performed well and showed a surprising growth result with a final OD_{600} of 2.22, which is very close to that of MRS, this result indicates that the HH is rich in nutriments, and the strain of *L. farciminis* found its requirements in this hydrolyzate. As expected, the glucose had a significant effect on *L. farciminis* growth and its addition to the HH (HH-G medium) enhanced significantly the strain growth, this may be explained by the fact that LAB need a carbon source for their growth (Zhang *et al.*, 2014). The lowest OD_{600} was observed in Y-G composed of glucose and yeast extract, in comparison with HH-G, this last induced a good growth potential, which is three-times superior than that obtained on Y-G. This result signifies that HH is more efficient than the yeast extract known to be rich in amino acids, nucleic acids and growth factors. To elucidate the effect of yeast extract supplementation, we tested the medium HH-G-Y, as a result, a significant difference was observed between growth on HH-G and HH-G-Y. The difference of 7.5% of growth due to the supplementation of yeast extract may not be considerable and could be negligible if we look to the cost of yeast extract as a medium component. Indeed, the remove of yeast extract could reduce significantly the medium cost, because this component has been reported to be very costly (Hujanen *et al*., 2001) and several studies aimed to replace it. As the aim of this study is to formulate a "low cost" medium for LAB growth, we preferred to retain HH-G as sheep Hooves Medium (HM), looking to its low cost composition (only glucose and HH) and its high performance to cultivate *L. farciminis*. This medium was used for the further experiments and was tested to support other strains growth.



Results are means of triplicate measurements.

Figure 1. Growth of *Lactobacillus farciminis* DSM20184 on MRS, Y-G medium and HH based media.

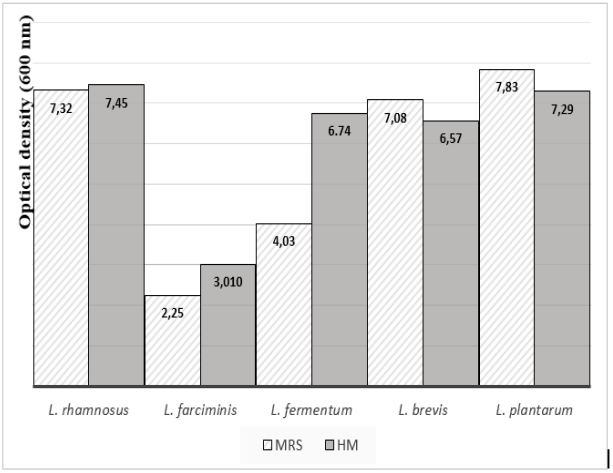


Figure 2. Maximal OD_{600} of the five strains reached on HM and the MRS.

Comparison of LAB strains growth on HM and MRS

A comparative study was carried out between this medium and the standard medium MRS to determine the efficiency of HM as growth medium for LAB. For this purpose, five LAB strains were grown on the two media and the growth parameters were compared (lag time, OD_{600} max and specific growth rate). OD_{600} max results are given in figure 2. In general, HM showed high performance to support the growth of the five strains. The most significant result was that of *L. fermentum*, this strain showed a high growth potential with a maximal OD_{600} of 6.74, which is 67.24% higher than that on MRS. Similarly, HH showed a good performance to cultivate *L. farciminis*, and the maximal OD600 surpassed that reached on MRS with 33.77%. L. rhamnosus sp. showed also a good growth potential (OD of 7.45) which is close to that observed on MRS but surpassed it significantly ($p<0.05$). The important growth results of *L. fermentum, L. farciminis* and *L. rhamnosus* obtained on HM indicate that this medium, composed only of HH and glucose, was sufficient to recover their requirements and could be used to enhance their biomass production. For *Lactobacillus brevis* and *Lactobacillus plantarum,* although OD_{600} max results on HM were important (6.57 and 7.29 respectively), those were slightly lower than those obtained on MRS. These results may be due to the origin of these two strains, selected originally from camel milk known to be rich in nutrients, hence, they may be more fastidious and require others nutrients not found in HM.

Eventually, the HM cultivated well the LAB strains, further experiments should be carried out testing the cultivation of other type strains, to define their requirements and to determine the components to be added to HM to standardize it as a growth medium for all LAB strains. In fact, MRS is appropriate for LAB growth but its composition was not based on their requirements, it was originally formulated to support the growth of some Lactobacilli growing imperfectly on tomato juice medium (de Man *et al*., 1960). Thereby, some MRS components are not necessary required by all LAB strains. For example, Horn *et al*., (2005) reported that nitrogen sources in MRS are in excess and are not incorporated into biomass, while the components used as carbon source were reported to be the critical ingredients in MRS medium for *Lactobacillus.* (Zhang *et al*., 2014).

Figure 3 represents the kinetics of growth of the five strains on HM and MRS. On HM, specific growth rates were calculated and compared to those obtained on MRS (table1). From the general view, the five strains showed normal growth curves, and in except of *L. fermentum*, lag time of the strains was long specially that of *L. plantarum* and *L. brevis* which presented similar growth curves. The five strains reached their maximal growth after about 18 to 20 h, their μ_{max} comparison showed that growth rates on HM were higher than those on MRS for *L. rhamnosus* and *L. brevis*, while *L. fermentum, L. farciminis* and *L. plantarum* had grown faster on MRS.

Generally, HM was efficient to cultivate the five strains and it gave significantly good results in comparison with those of MRS. From these results we can conclude that sheep hooves hydrolyzates is a very promising product, because, as reported by many studies, animal's by-products are known to be rich essentially in protein (Selmane *et al*., 2008), their hydrolysis could produce peptones of high quality which may serve for growth media formulation. In the same context, Aspmo *et al*, (2005) studied the use of cod viscera hydrolyzate for LAB growth, and

Table 1. Specific growth rate (μ_{max}: h^{-1}) of LAB strains reached on HM and MRS.

	L. farciminis	*L. fermentum*	*L. rhamnosus*	*L. brevis*	*L. plantarum*
HM	0.365	0.755	0.532	0.510	0.375
MRS	0.564	0.965	0.455	0.428	0.464

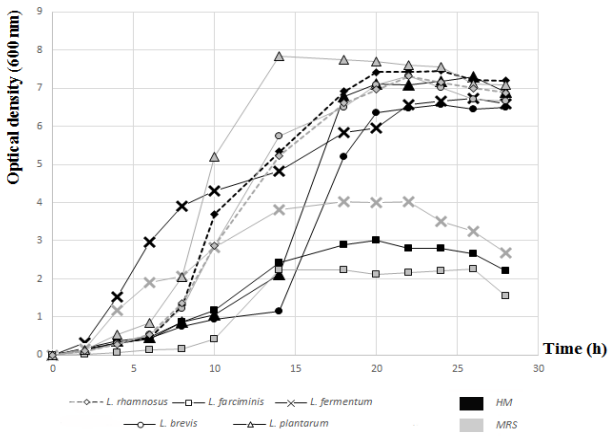


Figure 3. Growth kinetics of *L. fermentum, L. farciminis, L. brevis, L. plantarum* and *L. rhamnosus* on HM and MRS.

Kurbanoglu *et al.*, (2004) used ram horn peptones to cultivate *Lactobacillus casei*, these substrates showed good efficiency, which confirm the utility of animal's by-products hydrolyzates as supplements for biomass and lactic acid productions from LAB.

Conclusion

This study proposed the use of a slaughterhouse by-product known to be rich in protein to solve the problem of costly media components. Sheep hooves hydrolyzate had successfully replace the yeast extract used as a nitrogen source. In addition, HM composed only of HH and 20g L^{-1} of glucose showed a high performance to cultivate LAB strains especially *L. fermentum* DSM20049 and *L. farciminis* DSM20184. If applied, this way of exploitation will help for slaughterhouse waste management and will reduce environment pollution. Moreover, it may help for the reduction of LAB biomass production costs. Results found in this study are important and merit to be reinforced by optimizing medium for biomass and, probably, metabolites productions.

Acknowledgements

The work was financially supported by the laboratory of applied biochemistry and microbiology-University of Badji Mokhtar Annaba, Algeria. The authors would like to thank Pr. Nour Eddine Karam and the Pr. Abdeltif Amrane for the LAB strains.

References

Aspmo, S.I., Horn, S.J. and Eijsink, V.G.H. 2005. Use of hydrolysates from Atlantic cod (*Gadus morhua* L.) viscera as a complex nitrogen source for lactic acid bacteria. FEMS-Microbiology Letters 248: 65–68.

Belmessikh, A., Boukhalfa, H., Mechakra-Maza, A., Gheribi-Aoulmi, Z. and Amrane, A. 2013. Statistical optimization of culture medium for neutral protease production by *Aspergillus oryzae*. Comparative study between solid and submerged fermentations on tomato pomace. Journal of the Taiwan Institute of Chemical Engineers 44: 377–385.

Clausen, E., Gildberg, A. and Raa, J. 1985. Preparation and testing of an autolysate of fish viscera as growth substrate for bacteria. Applied and Environmental Microbiology 50: 1556–1557.

Coelho, L.F., De Lima, C.J.B., Rodovalho, C.M., Bernardo, M.P. and Contiero, J. 2011. Lactic acid production by new *Lactobacillus plantarum* LMISM6 grown in molasses: Optimization of medium composition. Brazilian Journal of Chemical Engineering 28: 27-36.

Cui, F., Li, Y. and Wan, C. 2011. Lactic acid production from corn stover using mixed cultures of *Lactobacillus rhamnosus* and *Lactobacillus brevis*. Bioresource Technology 102: 1831–1836.

De Man, J.C., Rogosa, M. and Sharpe, M.E. 1960. A medium for the cultivation of lactobacilli. Journal of Applied Bacteriology 23(1): 130–135.

Franz, C.M.A.P., Cho, G.S., Holzapfel, W.H. and Gálvez, A. 2010. Safety of Lactic Acid Bacteria. In Mozzi, F., Raya, R.R. and Vignolo, G.M. (Eds.). Biotechnology of Lactic Acid Bacteria: Novel Applications, p. 211-232. Iowa: Wiley-Blackwell.

Gaudreau, H., Renard, N., Champagne, C.P. and Horn, D.V. 2002. The evaluation of mixtures of yeast and potato extracts in growth media for biomass production of lactic cultures. Candian Journal of Microbiology 48: 626-634.

Georgiva, R., Koleva, D., Nikolova, D., Yankov, D. and Danova, S. 2009. Growth parameters of *Lactobacillus plantarum*, isolated from traditional white cheese. Biotechnology & Biotechnological Equipment 23: 861-865.

Horn, S.J., Aspmo, S.I. and Eijsink, V.G.H. 2005. Growth of *Lactobacillus plantarum* in media containing hydrolysates of fish viscera. Journal of Applied Microbiology 99: 1082–1089.

Hujanen, M., Linko, S., Linko, Y.Y. and Leisola, M. 2001. Optimisation of media and cultivation conditions for L (+) (S)-lactic acid production by *Lactobacillus casei* NRRL B-411. Applied Microbiology Biotechnology

56: 126–130.

Kassas, Z., Djeghri-Hocine, B., Hanoune, S., Derradji, Z., Boudour, A., and Boukhemis, M. 2015. Optimization of Split Pea and Dry Figs-Based Media for the Growth of *Lactobacillus plantarum* through Plackett-Burman and Response Surface Methodological Approaches. Pakistan Journal of Biological Sciences 18(3): 122-128.

Kurbanoglu. E. B. (2004) Enhancement of lactic acid production with ram horn peptone by *Lactobacillus casei*. World Journal of Microbiology and Biotechnology 20: 37–42.

LeBlanc, J.G., Taranto, M.P., Molina, V. and Sesma, F. 2010. B-Group Vitamins Production by Probiotic Lactic Acid Bacteria. In Mozzi, F., Raya, R.R. and Vignolo, G.M. (Eds.). Biotechnology of Lactic Acid Bacteria: Novel Applications, p. 341-359. Iowa: Wiley-Blackwell.

Nancib, A., Nancib, N., Meziane-Cherif, D., Boubendir, A., Fik, M. and Boudrant, J. 2005. Join effect of nitrogen sources and B vitamin supplementation of date juice on lactic acid production by *Lactobacillus casei* subsp. *rhamnosus*. Bioresource Technology 96: 63–67.

Roudj, S., Belkheir, K., Zadi Kram, H. and Karam, N. 2009. Protéolyse et autolyse chez deux lactobacilles isolés de lait camelin du Sud-Ouest Algérien. European Journal of Scientific Research 34: 218-227.

Safari, R., Motamedzadegan, A., Ovissipour, M., Regenstein, J.M., Gildberg, A. and Rasco, B. 2009. Use of hydrolysates from yellowfin Tuna *(Thunnus albacares)* heads as a complex nitrogen source for lactic acid bacteria. Food and Bioprocess Technology 5:73–79.

Selmane, D., Christophe, V. and Gholamreza, D. 2008. Extraction of proteins from slaughterhouse by-products: Influence of operating conditions on functional properties. Meat Science 79: 640–647.

Vazquez, J.A. and Murado, M.A. 2008. Enzymatic hydrolysates from food wastewater as a source of peptones for lactic acid bacteria productions. Enzyme and Microbial Technology 43: 66–72.

Zhang Y, Ng IS, Yao C, Lu Y (2014) Orthogonal array deciphering MRS medium requirements for isolated *Lactobacillus rhamnosus* ZY with cell properties characterization. Journal of Biosciences and Bioengineering 118(3): 298-304.