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Some Physiological and Biological Studies on Reuterin production from *Lactobacillus reuteri*

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Abstract : Lactic Acid Bacteria (LAB) can be used as food preservatives to improve food stability and safety. This is due to its ability to produce antimicrobial substances which can inhibit the growth of the food poisoning organisms. LAB produce antimicrobial compounds named bacteriocins. This study focused on bacteriocin named reuterin which produced from *Lactobacillus reuteri* strain and its optimal production condition. The metabolite *L. reuteri* bacteriocin (reuterin) was extracted and the antimicrobial activity was evaluated against some hospitalized bacterial and fungal pathogens. The reuterin producing *L. reuteri* exhibited the highest inhibition zone (22.2, 22.5 and 22.7 mm) against *E.coli, Staphylococcus aureus* and *Candida albicans*, respectively, when grown on optimized condition, i.e., growth on 2% glucose, soy bean (sb) or yeast extract as nitrogen source, all MRS salts medium and inoculated by 21 x10^8 cfu/ml, pH 6.5 at 37°C for 24hr anaerobically. This study gave us the possibility to use reuterin as food preservative to control pathogenic microorganisms and food spoilage.
Key Words: *Lactobacillus reuteri*, reuterin, inhibition zone, amikacin and fluconazole.

Introduction

It is clearly shown that human gut bacteria consists of approximately 10^4 colony forming unit (cfu)/ml and about 500 to 1000 different species and live in their host in symbiosis. This bacterial population is controlled by diets[1].

From few years ago, scientists discovered beneficial bacteria in yogurt, pills & other food and called it probiotics. The Latin & a Greek word "probiotic" means "favorable to life". According to the Food & Agricultural Organization (FAO)[2]; "they are any living microorganisms administrated in adequate amounts which have a beneficial health effect on their host."

According to Fuller[3], the probiotics can be defined as "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal *microbial balance." Recently, The word ''probiotic'' means a large numbers of* microorganisms, mainly bacteria (as Lactic acid bacteria (LAB)& non lactic acid bacteria) and few yeasts whom can stay a live until reach to the colon and then improve the host health by its beneficial actions on its host. For example, LAB (e.g. *Lactobacillus reuteri*, *Lactococcus acidophilus*, *Enterococcus faecium*, etc.) and non-lactic acid bacteria (e.g. *Escherichia coli*) also some yeasts (e.g. *Saccharomyces cerevisiae,)* are also considered as probiotics.

These LAB are Gram-positive rods and usually live an-aerobically but in few cases, they can grow also on aerobic conditions[4,5].

Lindgren and Dobrogosz[6] stated that LAB can preserve dairy foods by producing some organic compounds that are antagonistic to other microorganisms. Among these compounds, the bacteriocins which are short chain proteins and have strong ability to inhibit the pathogens growth[7 & 8]. Many researches had focused on using bacteriocins as food preservatives & control of human bacterial pathogens and disease appearance. The probiotic activity of these bacteria is studied and recorded by Gilliand[9]; Cleveland *et al*., [10]; Mojgani & Ashtiani [11]and Diez-Gonzalez[12].

Bacteriocins production can be influenced by many factors, including medium composition[13] and environmental factors[14,15]. De Man Rogosa and Sharpe (MRS) medium is the standard, specific culture medium for (LAB), but it is not suitable for industrial-scale production due to its high cost. MRS had used for fermentation, while the purpose was to produce bacteriocins, lactic acid, some enzymes, or other metabolites [16,17]. The lowering of the culture media cost have been published [18,19,20], not referring to industrial fermentation. The aim of this study was to increase the production and efficiency of the studied reuterin and reduce the fermentation cost to increase the industrial production rate.

Materials and Methods:

Bacterial and Fungal Strains

Lactobacillus reuteri (*L. reuteri)* which is Gram-positive bacillus bacterium kindly provided from Chr. Hansen's Lab. Inc., Denmark. On other hand, Pathogenic strains, *Escherichia coli*, and *Staphylococcus aureus* in addition to Candida albicans as yeast fungi were obtained from the clinical lab. of the El Demerdash Hospital in Cairo, Egypt and they are multi drug resistant (MDR) isolates.

Positive control of antibiotics: It´s include Amikacin (32 µg) as antibacterial antibiotic and Fluconazole (150 µg) as antifungal antibiotic derived from pfizer pharmaceutical company, Cairo, Egypt.

Sugar cane molasses by product obtained from "The Sugar and Integrated Industries Co.", El Hawamdia, Giza, Egypt.

Corn steep liquor (CSL) by product was obtained from Egyptian Starch and Glucose Company, Moustoroured, Cairo, Egypt.

Soybean meal -extract (SBE) prepared by aqueous extraction (at 121ºC for 20 min.) of a commercial sample of soybean seeds (solid/liquid ratio 1/20, w/v) followed by filtration and freeze drying of the resulted extract.

Gas Generating Kits used for generation of hydrogen and carbon dioxide in metal or plastic anaerobic jars. It was purchased from Oxoid Ltd, Basingstoke, Hampshire, England.

Culture Media:

a- Nutrient agar medium was used for the growth and maintenance of the pathogenic strains *Escherichia coli* and *Staphylococcus aureus*[21].

b- MRS medium (De Man- Rogosa- Sharp- medium): according to De Man, et al.,[22], this medium was used for the growth and maintenances of the probiotic *L. reuteri,* it was prepared broth (without addition of agar) and can be used for determination of the growth density of the investigated probiotic and its bacteriocin production.

c- Potato Dextrose Agar (PDA): W**as** used for the growth and maintenances of the yeast strain *Candida albicans*[23].

Isolation and purification of bacteriocin:

Variations in the level of reuterin production were evaluated by the well diffusion assay [24]and using cork pore diameter 5 mm. Experiments were carried out in duplicate. 0.1 % (v/v) of *L.reuteri* was inoculated at

a final concentration of about 10^8 CFU/ml according to Emanuel Goldman, Lorrence H Green[25]. The antimicrobial activity of the supernatants was evaluated by the critical dilution assay of Barefoot & Klaenhammer[26] with using of Amikacin (32 µg) as antibacterial antibiotic and Fluconazole (150 µg) as antifungal antibiotic. Reuterin activity was defined as the reciprocal of the highest dilution showing definite inhibition of the indicator strains and was expressed as activity units per milliliter (AU/ml).

Determination of the minimum inhibitory concentration (MIC) of *L.reuteri* supernatent on indicator pathogens:

According to Andrews *et al*., [27], minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial metabolite that inhibits the visible growth of a microorganism after overnight incubation. Supernatants obtained from *L. reuteri* bacterium was used to determinethe MIC of each by using the original supernatant of each as follow: 0.025, 0.05, 0.10, 0.15, 0.20 and 0.25 ml. Well-cut diffusion technique was used to determine the minimal inhibitory effect of the previous dilutions against the indicator pathogens.

Preparation of cell-free culture supernatants:

Cell-free culture supernatants (CFCS) were obtained by centrifugation at 5000 rpm for 20 min of *L. reuteri* culture grown under specific cultivation conditions under study.

Optimization studies:

It include incubation periods, inoculum size, incubation temperature ranged from 25-45ºC and initial pH ranged from 5.0 to 8.5. Effect of various carbon sources, nitrogen sources and medium salt elimination were evaluated in relation to reuterin production.

Data Analysis:

The recorded data and results were summarized and analyzed in the tables according to Coulombier D. *et al,* [28].

3- Results

3.1. Effect of using different Incubation times:

Table (1) clearly shown that the reuterin production by *L. reuteri* being affected by incubation time, it reached a maximum at 24 & 48 hr. which was 19.0 mm inhibition zone for *E. coli* and 22.0 mm for both *Staph. aureus* or *Candida albicans* comparing to positive control(Amikacin 32µg/ml as antibacterial agent & Fluconazole 150 µg/ml as antifungal agent) in addition to sterile distilled water as negative control. So 24hr. is the best incubation time for reuterin production, there for we select 24 hr. incubation period for reuterin production.

Table (1): effect of using different incubation periods on reuterin production

Incubation period (hr)	**Cell biomass g/l**	**Final pH**	**inhibition zone (mm)**		
			E.coli	***Staph.aureus***	***Candida albicans***
24	2.1	5.6	19.0	22.0	22.0
48	2.3	5.6	19.0	22.0	22.0
72	3.5	5.5	18.0	20.0	20.0
96	3.5	5.5	16.0	17.0	17.0
Amikacin 32 µg/ml			20.0	20.0	-----
Fluconazole 150 µg/ml			------	-------	18.0

3.2. Effect of using different initial pH values:

According to the nature of the studied organism which is acidophilic, the reuterin production by *L. reuteri* was highly affected by the initial pH values of the media. Eight pH values were selected to study their effects on reuterin production ranged from 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5. All environmental and nutritional factors were fixed and incubated for 24 hr.

Table (2) clearly illustrate that the pH 6.5 and 7.0 were the best pH values for maximum reuterin production than that of others under the same experimental conditions.

The maximum production of reuterin is after 24 hr. & pH 6.5 gave 19.0 mm inhibition zone for *E.coli* and 22.0 mm for both *Staph. aureus* or *Candida albicans comparing to positive control* (Amikacin 32µg/ml as antibacterial agent & Fluconazole 150 µg/ml as antifungal agent).

Table (2): the effect of using different pH values on reuterin production.

pH value	Cell biomass g/l	Final pH	inhibition zone (mm)		
			E.coli	***Staph.aureus***	***Candida albicans***
5.0	1.8	5.0	17.0	16.0	16.0
5.5	1.8	5.0	18.0	18.0	18.0
6.0	2.0	5.5	18.0	18.0	18.0
6.5	2.1	5.5	19.0	22.0	22.0
7.0	2.7	6.0	19.0	21.0	21.0
7.5	2.0	6.3	17.0	18.0	16.0
8.0	1.4	7.0	11.0	11.0	10.0
8.5	1.0	7.1	7.0	8.0	6.0

3.3. Effect of using different Incubation Temperatures:

At this experiment, the inoculated test tubes will incubated at 25, 30, 37 and 45 °C for 24 hr. and pH 6.5 with fixation of other environmental and nutritional factors and the cell biomass, final pH and reutrin productivity were recorded and listed in table (3).

The recorded data show that the reuterin productivity was clearly affected by the temperature of incubation. The reuterin productivity was maximized at 30 - 37 °C while at elevated temperature (45 °C) or low temperature (25 °C) the activity decreased. At 25 °C the activity decreased to 19 mm inhibition zone diameter while at high temperature (45 °C) there was no activity.

Table (3) : the effect of using different incubation temperatures on reuterin production.

Incubation temperature °C	Cell biomass g/l	Final pH	inhibition zone (mm)		
			E.coli	*Staph.aureus*	*Candida albicans*
25	1.8	5.0	19.0	19.0	19.0
30	1.8	5.0	19.0	22.0	22.0
37	2.1	5.5	19.0	22.0	22.0
45	0.4	6.3	-ve	-ve	-ve

-ve : negative (no reuterin productivity).

3.4. Effect of using different inoculum sizes:

Table (4) clearly illustrate the effect of using different inoculum sizes (3, 6, 9, 12, 15, 18, 21, 24 or 27 x 10^8 CFU/ml) on reuterin production with the fixation of all other environmental or nutritional factors according to the best final condition. According to the table (4), the best inoculum size gives the highest reuterin production was 21 x 10^8.

Table (4): the effect of using different inoculum sizes on reuterin production.

Inoculum size x10^8 cfu/ml	Cell biomass g/l	Final pH	inhibition zone (mm)		
			E.coli	*Staph.aureus*	*Candida albicans*
3	1.8	5.8	17.0	18.0	17.0
6	1.9	5.8	18.0	18.0	18.0
9	1.9	5.7	18.0	20.0	19.0
12	2.1	5.5	19.0	20.0	20.0
15	2.1	5.5	19.0	20.0	20.0
18	2.3	5.5	19.0	22.1	22.0
21	2.5	5.5	22.2	22.5	22.8
24	2.5	5.5	22.2	22.5	22.7
27	2.3	5.0	22.2	22.4	22.7

3.5. Effect of using different carbon sources:

Carbon source is the most important nutritional factor for both growth and reuterin production of *L. reuteri* strain. This study discusses the effect of using of different carbon sources instead of 2 % glucose of the reuterin production medium. Carbon sources include; fructose, lactose, sucrose or cane sugar molasses in a concentrations raged from 1 - 4%. Table (5) show that the best carbon sources were glucose (2%) and lactose (2%) followed by sucrose while fructose gave weak results on other hand, cane sugar molasses was not suitable for reuterin production (negative results).

Table (5): the effect of using different carbon sources with different concentrations on reuterin production.

Carbon Source	Cell biomass g/l	Final pH	inhibition zone (mm)		
			E.coli	*Staph.aureus*	*Candida albicans*
glucose					
1 %	1.5	6.0	18.0	18.0	18.0
2 %	2.5	6.0	22.2	22.5	22.8
3 %	3.1	5.5	18.0	20.0	20.0
4 %	3.4	5.5	17.0	18.0	16.0
fructose					
1 %	1.2	6.0	10.0	10.0	10.0
2 %	1.3	5.5	11.0	10.0	9.0
3 %	1.3	5.5	10.0	10.0	9.0
4 %	1.3	5.5	10.0	10.0	9.0
Sucrose					
1 %	1.6	6.0	16.0	16.0	16.0
2 %	1.9	6.0	17.0	20.0	20.0
3 %	2.4	5.5	17.0	18.0	17.0
4 %	2.5	5.5	17.0	16.0	16.0
lactose					
1 %	1.5	6.0	18.0	19.0	20.0
2 %	2.5	6.0	19.2	22.0	22.1
3 %	3.1	5.5	18.1	20.0	20.0
4 %	3.4	5.5	17.1	18.0	16.2
Cane sugar molasses					
1 %	0.25	5.0	-ve	-ve	-ve
2 %	0.25	5.0	-ve	-ve	-ve
3 %	0.30	5.0	-ve	-ve	-ve
4 %	0.30	5.0	-ve	-ve	-ve

-ve : negative (no reuterin productivity)

3.6. Effect of using different Nitrogen sources:

six nitrogenous compounds were used instead of nitrogenous component of the reuterin production medium, at fixed optimum conditions (i.e.: at pH 6.5, temperature 37 °C, inoculum size 21 x 10^8 with 2% glucose). These nitrogen sources were; peptone, yeast extract, beef extract, casein hydrolysate, SBE and CSL (according to the equivalent N-basis). Table (6) showed that the most favourable nitrogenous compound was yeast extract which gave the highest activity expressed by inhibition zone diameter (The maximum production of reuterin is 22.2 mm inhibition zone for *E.coli* and 22.5 mm for *Staph. aureus* and 22.8 mm for *Candida albicans comparing to positive controls*) followed by corn steep liqour which gave almost the near same reuterin productivity. Results of table 6 revealed that there is inversely proportionality between reuterin productivity and the cell biomass i.e.; the reuterin production will decrease when the cell biomass increased (and vice versa).

Table (6): effect of using different nitrogen sources on reuterin production (expressed as inhibition zone diameter)

Nitrogen Source	Cell biomass g/l	Final pH	inhibition zone (mm)		
			E.coli	*Staph.aureus*	*Candida albicans*
peptone	1.5	6.0	18.0	18.0	18.0
Yeast extract	2.5	5.5	22.2	22.5	22.8
Beef extract	3.1	5.5	17.0	18.0	18.0
Casein hydrolysate	3.4	5.5	17.0	19.0	19.0
Soy bean	5.8	6.7	11.0	10.2	10.1
Corn steep liquor	3.0	6.5	22.2	22.5	22.7
Control*	2.1	5.6	19.0	22.0	22.0

Control*: standard MRS broth medium.

3.7. Effect of salt elimination:

The results illustrated in table (7) showed that elimination of one salt resulted in decrease of reuterin productivity, so the reuterin productivity resulted from original MRS medium is highly affected by the culture medium component and elimination of one salt will decrease the reuterin productivity. So, all the medium salts are important for reuterin production with concentrations as in MRS medium.

Table (7): Effect of salt elimination on reuterin productivity

Eliminated salt	Cell biomass g/l	Final pH	inhibition zone (mm)		
			E.coli	*Staph.aureus*	*Candida albicans*
K_2HPO_4	1.5	5.5	10.0	12.0	12.0
Sod. Acetate trihydrate	3.1	5.5	10.0	12.0	11.0
Triammonium citrate	3.4	5.5	10.0	14.0	11.0
$MgSO_4$ 7 H_2O	5.8	6.7	17.0	17.2	18.1
$MnSO_4$ 4 H_2O	3.6	6.5	18.0	19.0	19.1
Control*	2.1	5.6	19.0	22.0	22.0

Control*: standard MRS broth medium.

3.1.4.2 The Minimum Inhibitory Concentration (MIC) Test:

The results of table (8) show that, the best volume for culture filtrate (free of cells) containing reuterin is 0.05 ml, below which negative effect of reuterin against studied bacterial and yeast strains was recorded and then it can be considered as the MIC. On the other hand, the volume 0.15 ml as well as 0.2 ml and 0.25 ml of

culture filtrate were the best concentrations gives the highest reuterin production and antimicrobial activity against all studied pathogenic strains.

Table (8): The effect of using different culture filtrate concentrations on reuterin production as inhibition zone diameter (mm)

Culture filtrate conc.(ml)	*Escherichia coli* 24h	*Staphylococcus aureus* 24h	*Candida albicans* 24h
0.025	-ve	-ve	-ve
0.05	10.0	10.0	12.0
0.10	19.0	22.0	22.0
0.15	22.2	22.5	22.7
0.20	22.2	22.5	22.7
0.25	22.2	22.5	22.7

-ve : negative (no reuterin productivity).

Discussion:

This study focused on the effect of two growing factors; environmental and nutritional factors. The effect of environmental factors on reuterin production, including incubation periods, pH values, incubation temperatures and inoculum size were studied by many researchers[29]. On other hand,the nutritional factors (medium composition) play an important role in bacteriocin production[30]. Furthermore, reuterin production is strongly affected by medium composition [31]. Our study showed that, the pH values ranged from 6.5 to 7.0 were the optimum pH values gave the highest reuterin productivity with moderate cell biomass of the studied *L .reuteri*. Above or bellow this pH value will lead to decrease in reuterin productivity. These results agree with Leroy and De Vuyst [32], they proved that the bacteriocin activity of *B. bifidum* was lower at pH ranging from 7.5 to 8.0 and from 5.5 to 6.5.

Generally, reuterin production by *L. reuteri* was considered as a temperature sensitive process. Furthermore, the optimal growth temperature definitely not as like as the optimal bacteriocin production temperature[33] and it consistent with our results which exhibited a temperatures ranged from (30–37°C) were optimum for reuterin production while temperature 37°C only is optimum for cell growth. A higher production of bacteriocin in the range of temperature for optimal strain development has been observed in many LAB strains, such *as E. faecium* RZS C5 [32], *Lactobacillus sakei* Lb 706 [24]. However no significant differences were reported for bacteriocin production at 30 and 37°C, which may prove that growth temperature in that range does not play a defining role in bacteriocin activity and it coincide with Todorov and Dicks [34] with regards to *L. plantarum* bacteriocins.

The studying of using different nutritional factors on reuterin production aimed to attain higher reuterin yields with very low cost media as food industry wastes (e.g. sugar cane molasses & CSL) or low cost proteins as SBE. With regards to studied carbohydrates, low reuterin production was produced when using sucrose and fructose while sugar cane molasses does not initiate reuterin production for the studied strain, it may be due to that the studied organism did not have the enzymatic system responsible for the digestion of sugar cane molasses. Since fructose and molasses reduced cell biomass and reuterin productivity, we deduced that the studied probiotic could not utilize these saccharides as a sole carbon source, on the other hand, the sole carbon sources which were readily to be utilized and gave high reuterin productivity were glucose, lactose followed by sucrose. Bing, *et al*., [35]observed that the glucose concentration over 2.0 % could reduce the reuterin yield. On the other hand, the nitrogenous compound SBE reduces markedly the reuterin production but the cell growth was too high. It explain that the studied probiotic used the SBE to produce cells only not for reuterin production while CSL gave high reutern production (more than the control medium) with moderately high cell growth, this agree with Helal *et al*., {36}, although several researchers observed that higher bacteriocin production were recorded when increasing the concentrations of nitrogen content of the medium[37, 38].

Salt elimination test explain clearly that all medium salts needed for high reuterin production in a different degrees and the less effective one was $MnSO_4$ 4 H_2O which gave slight effect.

Finally, our study tested the effect of reuterin concentrations in the culture filtrate on the studied pathogens, it was found that the MIC for activity is 0.05 ml of the reuterin below which there is no antimicrobial activity and concentration 0.15 ml of the culture filtrate is the optimal concentration gave the best antimicrobial activity, this results agree with Helal *et al.*, [36].

Conclusion:

This study revealed that the reuterin productivity and cell biomass of the studied *Lactobacillus reuteri* is highly sensitive to environmental and nutritional factors which affecting on the studied probiotic. The relation between the cell biomass and reuterin productivity is not in linear relationship i.e.; reuterin productivity is optimized with moderate cell growth while increasing the cell biomass did not affecting on the reuterin productivity.

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References:

1. Lee, Y. K. and Salminen, S. (2009). Handbook of probiotics and prebiotics. 2nd ed. Hoboken, N.J.: John Wiley & Sons, Inc.
2. FAO/WHO. 2001. Evaluation of health and nutritional properties of powder milk and live lactic acid bacteria. Food and Agriculture Organization of the United Nations and World Health Organization Expert Consultation Report.
3. Fuller, R. (1989). Probiotics in man and animals. J.Appl. Bact. 66, 365-378.
4. Holzapfel, W.H., Haberer, P., Geisen, R., BjÖrkroth, J. and Schillinger, U. (2001). Taxonomy and important features of probiotic microorganisms in food and nutrition. American Journal of Clinical Nutrition 73 (2 Suppl.), 365S–373S.
5. Anal, A. K. and Singh, H. (2007). Recent advances in microencapsulation of probiotics for industrial application and targeted delivery.Trends Food Sci. Technol., 18,240–251.
6. Lindgren, S.E., and Dobrogosz, W.J. (1990). Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiol. Rev. 87, 149-164.
7. Ruiz-Barbara, J.L., Cathcart, D.P., Warner, P.J. and Jimmenez-Diaz, R. (1994). Use of *L.plantarum* LPCO10, a bacteriocin producer as a starter culture in Spanish style green olive fermentations. Appl .Environ. Microbiol. 2059-2064.
8. Benkerroum, N., Ghouati, Y. and Ghalfi, H. (2007). Screening for bacteriocin producing LAB from various Moraccan food products and partial characterization of putative bacteriocins. Biotechnol. 4:481-488.
9. Gilliand, S.E. (1990). Health and nutritional benefits from lactic acid bacteria. FEMS Microbiol. Rev. 87: 175-178.
10. Cleveland, J., Montville, T.J., Nes, I.F. and Chikindas, M.L.(2001).Bacteriocins: safe, natural antimicrobial for food preservation. Int. J. Food Microbiol. 71:1-20.
11. Mojgani, N. and Ashtiani, M.P. (2006). In vitro inhibition of *mastitis* pathogens by bacteriocin RN 86 produced by an indigenous strain of *L.casei* RN 86. J Appl Sciences .6: 2629-2634.
12. Diez-Gonzalez, F. (2007). Applications of bacteriocins in livestock. Curr. Issues Intestinal . Microbiol. 8, 15–24.
13. Zhou, X.X., Pan, Y.J., Wang, Y.B. and Li, W.F. (2008). Optimization of mediumcomposition for nisin fermentation with response surface methodology. J. Food Sci., 73: 245-249.
14. Leal-Sánchez, M.V., Jiménez-Díaz, R., Maldonado-Barragán, A., Garrido- Fernández, A. and Ruiz-Barba, J.L. (2002). Optimization of bacteriocin production by batch fermentation of *Lactobacillus plantarum* LPCO10. Appl. Environ. Microbiol. 68: 4465-4471.

15. Motta, A.S. and Brandelli, A. (2008). Evaluation of environmental conditions for production of bacteriocin-like substance by *Bacillus* sp. Strain P34. World J. Microbiol. Biotechnol. 24: 641-646.
16. Hummel, W., Schütte, H. and Kula, M.R. (1983). Large scale production of D lactate dehydrogenase for the stereospecific reduction of pyruvate and phenylpyruvate. Eur.J. Appl. Microbiol. Biotechnol. 18: 75-85.
17. Lu, Z., Breidt, F., Fleming, H.P., Altermann, E. and Klaenhammer, T.R.(2003). Isolation and characterization of a *Lactobacillus plantarum* bacteriophage, JL-1, from a cucumber fermentation. Int. J. Food Microbiol., 84: 225-235.
18. Dominguez, A.P.M., Bizani, D., Cladera-Olivera, F. and Brandelli, A. (2007). Cerein 8A production in soybean protein using response surface methodology. Biochem. Eng. J. 35: 238-243.
19. Trinetta V, Rollini M, Manzoni M (2008). Development of a low cost culture medium for sakacin A production by *L. sakei*. Process Biochem., 43: 1275-1280.
20. Wiese, B., Bru, E., Juárez, Tomás, M.S. and Nader-Macías, M.E.F.(2010). Optimization of low-cost culture media for the production of biomass and bacteriocin by a Urogenital *Lactobacillus salivarius* strain. Probiotics and Antimicro. Prot. 2: 2-11.
21. American Public Health Association, American Chemical Society, Association of Official Agricultural Chemists (1920). Standard Methods for the Examination of Water and Sewage. American public health association. p. 95.
22. De Man, J.D.; Rogosa, M. and Sharpe, M.E. 1960. A medium for the cultivation of lactobacilli. J. appl. Bact., 23: 130-135.
23. Harold Eddleman, Ph. D (February 1998). "Making Bacteria Media from Potato". Indiana Biolab. disknet.com. Retrieved 2011-03-04.
24. Schillinger, V. and Luke, K.K. (1989). Antibacterial activity of *Lactobacillus sake* isolated from meat. Appl.Environ. Microbiol. 55: 1091-1096.
25. Emanuel Goldman, Lorrence H Green (24 August 2008). Practical Handbook of Microbiology, Second Edition (Google eBook) (Second ed.). USA: CRC Press, Taylor and Francis Group. p. 864. ISBN 978-0-8493-9365-5. Retrieved 2014-10-16.
26. Barefoot, S.F. and Klaenhammer, T.R. (1983). Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl. Environ. Microbiol. 45:1808-1815.
27. Andrews, J.M. (2001). Determination of minimal inhibitory concentrations. J. Antimicrob. Chemoth. 48: 5-16.
28. Coulombier, D., Fagan, R., Hathcock, L. and Smith, C., (2001). Epi Info 6 Version 6.04.A Word Processing, Database and Statistical Program for Public Health. CDC, Atlanta, Delaware, USA.
29. Delgado, A., López, N.A.L., Brito, D., Peres, C., Fevereiro, P. and Garrido- Fernández, A. (2007). Optimum bacteriocin production by *Lactobacillus plantarum* 17.2b requires absence of NaCl and apparently follows a mixed metabolite kinetics. J. Biotechnol. 130: 193-201.
30. Li, C., BaI, J.H., Cai, Z.L. and Ouyang, F. (2002). Optimization of a cultural medium for bacteriocin production by *Lactococcus lactis* using response surface methodology. J. Biotechnol., 93: 27-34.
31. Ganzle, M. G., Weber, S. and Hammes, W. P. (1999) Effect of ecological factors on the inhibitory spectrum and activity of bacteriocins. International Journal of Food Microbiology. 46: 207-217.
32. Leroy, F., and De Vuyst, L. (2002). Bacteriocin production by *Enterococcus faecium* RZS C5 is cell density limited and occurs in the very early growth phase. International Journal of Food Microbiology. 72: 155–164.
33. Leroy, F., and De Vuyst, L. (1999b). The presence of salt and a curing agent reduces bacteriocin production by *Lactobacillus sakei* CTC 494, a potential starter culture for sausage fermentation. Applied and Environmental Microbiology. 65: 5350–5356.
34. Todorov, S.D. and Dicks LMT. (2005). Growth parameters influencing the production of *Lactobacillus rhamnosus* bacteriocins ST461BZ and ST462BZ. Ann. Microbiol. 55(4): 283-289.
35. Bing Han, Zhanqiao Yu, Baosheng Liu, Qingshan Ma and Rijun Zhang. (2011). Optimization of bacteriocin production by *Lactobacillus plantarum* YJG, isolated from the mucosa of the gut ofhealthy chickens. African Journal of Microbiology Research .5(10): 1147-1155.
36. Mohamed, M.I.Helal, Nayera, A.M. Abdelwahed , Aza. M. Abdel-Fattah and Madeha, O.I.Ghobashy. Studies on the production of antimicrobial substances produced from *Bifidobacterium bifidum.* J. of applied science research, 9(3): 1554-1563, 2013.

37. Aasen, I.M., Moretro, T., Katla, T., Axelsson, L. and Storro, I. (2000). Influence of complex nutrient, temperature and pH on bacteriocin production by *Lactobacillus sakei* CCUG42687. Appl. Microbiol. Biotechnol. 53:159- 66.
38. Guerra, N.P. and Pastrana, L., (2001). Enhanced nisin and pediocin production on whey supplemented with different nitrogen sources. Biotechnol. Lett. 23, 609-612.

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