A novel growth strategy for propagation and bacteriocin production of *Lactobacilli*

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Introduction

Lactobacillus a bacterial group belonging into the genre of Lactic Acid Bacteria (LAB). Their metabolic end products such as lactic acid, acetic acid and protein structure antimicrobial compounds called bacteriocins are widely applied as food preservatives in the contemporary food industry.(1) *Lactobacillus* distinctive ability is to decompose complex carbohydrate sources into simpler forms and synthesise mainly lactic acid. Numerous *Lactobacillus* strains are producing antimicrobial compounds called bacteriocins. These substances are divided into three major groups. The plurality of the discovered substances belongs into the first group named Lantibiotics. Lantibiotics are mainly active against bacteria of the same genus and they are produced during growth of the bacilli, especially during late exponential phase. They obtain a low molecular weight at about 3.000- 4.500 d approximately.(5.)

Lantibiotics are thermostable molecules, can withstand acidic conditions occurring during growth and they can be degraded by the enzymes existing in the gastrointestinal tract. They are considered safe for consumption from human beings, having no toxic effect on human health. Their use as natural antimicrobial preservatives has been proposed throughout the recent years. Several methods for their introduction into food have been examined in a constant effort to replace the chemical and in general artificial methods of food preservation due to high health risks resulting from their usage.The most common and widely exploited worldwide lantibiotic is nisin, which is commercially available. Its use as a natural preservative has been approved in the USA and in Europe. In this work an attempt to develop a simplified low molecular weight nutrient medium for Lactobacilli growth and bacteriocin production has been made. Although Lactobacilli are widely applied in modern food industry their potential as natural anticontaminants has not been deeply exploited. (3) The scope ogf this project is primarily to develop a novel growth strategy for bacteriocin production.

Materials and Methods

Inoculum source

All the Lactobacilli, Lactobacillus casei NCIMB 11970 Lactobacillus plantarum NCIMB 8014, Lactobacillus lactis NCIMB 8586 and the target strain Lactobacillus delbruckii subsp.lactis 8117 were provided in a lyophilised form by National Collection of Food and Marine bacteria(NCIMB) , Aberdeen , Scotland.

Preliminary Growth experiments

Pyrex glass pressure tubes sealed with butyl rubber stoppers and aluminium seals were used to test the effect of basal and optimum on Lactobacilli growth . The tubes were prepared under aseptic and anaerobic conditions. The media recipe for the basal medium is glucose 2% w/v, yeast extract 1.5% w/v, peptone 1% w/v, sodium acetate, 0.5% w/v,tri-sodium citrate 0.2%, potassium hydrogen phosphate 0.2% w/v, MgSO□ 0.02% w/v , MnSO□ 0.002% w/v and resazurin dye 0.0005% v/v. Each component was tested separately so to certify its influence on growth in a range of concentrations between 0% w/v to 4% w/v.All materials where bought from Sigma-Aldrich, UK All the components were combined and an optimised medium was fabricated. The medium's recipe is glucose 2% w/v, yeast extract 2 w/v, sodium acetate, 1% w/v w/v,tri-sodium citrate 1% w/v, potassium hydrogen phosphate 0.5 w/v and resazurin dye 0.0005% v/v.

Ultrafiltration

A bench membrane apparatus (stirred cell unit, Amicon 8200) was used for the filtration of the media. The reactor system was composed of an ultrafiltration stirred cell unit of 200 ml maximum process volume, a magnetic stirrer and an effective area of 28.7 cm². (Millipore Co., UK) and able to withstand operating conditions of maximum pressure 75 psi (approximately 5.17 bar) , maximum temperature of 85ºC and a pH range 2-10. The stirrer speed was set at 500 rpm through the series of experiments concerning the nutrient media filtration. The molecular weight cut-off (MWCO) of ultrafiltration polysulphone membranes in use was 30 kDa and 4 kDa. The filters were provided from Millipore Co., UK, (30 kDa) and from Microdyn-Nadir Co. (4 kDa), All the membranes were left for 24h soaking in distilled water to ameliorate diffusivity of the molecules. The pressure in the system was controlled by an electronic pressure adjustment value fitted with a pressure gauge. The cell unit was pressurizes by constant compressed nitrogen at 200 kPa. The operating temperature was controlled at 25ºC constantly by connecting via rubber tubes the cell unit water jacket with a water bath (Grant Water bath, UK). The stirred cell unit was operated in a batch dead-end mode.

Measurement of cellular growth and biomass

The cellular growth was measured by placing the pressure tubes into a spectrophotometer fitted with a test tube holder (PU 8625 UV/VIS Philips, France) at 660 nm. The tube had a 1.8 cm. light path.

Assay for nisin and bacteriocin concentration and quantification

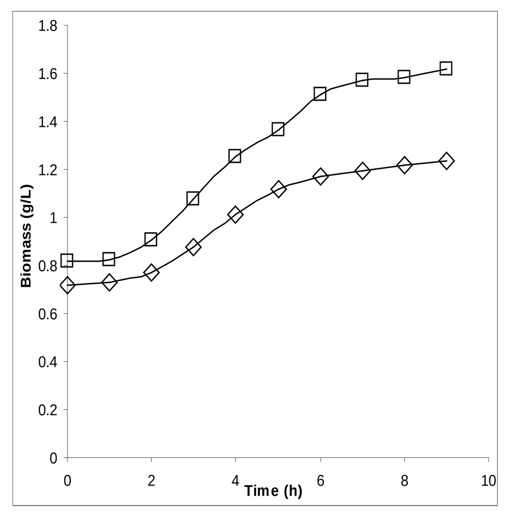
Lactobacillus delbruckii subsp.lactis 8117 was selected as the target strain. A consistent inoculum size was prepared. The amount of the bacteriocin produced by each under investigation strain was primarily defined on the samples taken during the pH and temperature controlled fermentations. Furthermore an analysis of the overall amount of bacteriocin produced was performed on the broths collected after the end of fermentation. The selected samples (pH fermentation at 6.5) were transferred into 10 ml conical plastic tubes (Fisherbrand, UK) and centrifuged (10.000 rpm for 15 min.) (Biofuge Stratos Sorall, Kendro Products, Germany) in order to remove completely the biomass. The clarified liquid was filtrated through a 0.2 μm pore size filter for sterilisation. The sterilised liquid's pH was adjusted at 6.0 to eliminate the antimicrobial effect of hydrogen peroxide and lactic acid and then it was diluted with fresh medium. Into 25 ml of 0.02 M of HCl 25mg of nisin are dispersed. This solution equals to 1000 IU of Nisin. According to this formula the necessary quantities of solid nisin were calculated to fabricate standard solution at the following concentrations: 0, 25, 50, 75, 85, 100, 110, 125, 150, 175, 200, 250, 500, 750, 1000, 1250, 1500, 1750, 2000 IU/ml. The solutions are preserved stable (up to 30 days) into 4°C. Into glass tubes containing 8 ml of optimised medium including metals ,so to ensure that any effect on growth of the tested microorganism results from the bacteriocin produced and not due to any other factors such as nutrient exhaustion of optimum anaerobic medium for the growth of the tested strain L.delbruckii (medium recipe:2% glucose,2% Y.E., 1% sodium acetate, 1% tri-sodium citrate, 0.5% KH2PO4, 0.05% magnesium sulphate, 0.005% manganese sulphate) 1 ml of the frozen inoculum of L.delbruckii and 1 ml of the supernatant resulting from pH control fermentations of differential concentration is added.

Results and discussion

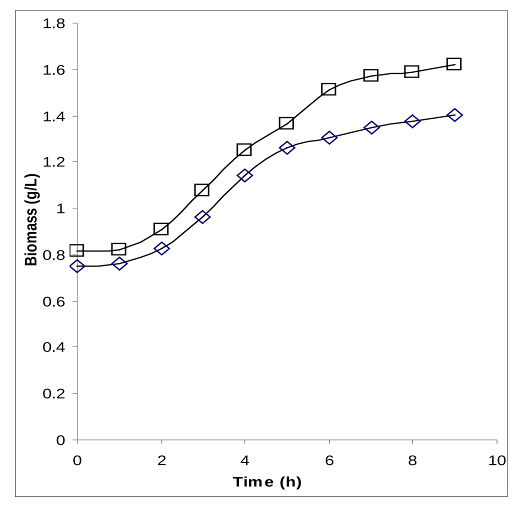
Bacteriocin production on filtrated media

Primarily the effect of commercially available nisin was tested. The effect was tested against the selected *Lactobacilli* target strain *L.delbruckii subsp.lactis NCIMB 8117* in a range of 0 IU/ml to 2000 IU/ml. A dose response curve was achieved based on the maximum growth rates achieved under the testing of each concentration against the target strain. *L.delbruckii* has a growth rate 0.32 and doubling time of 2.15h.

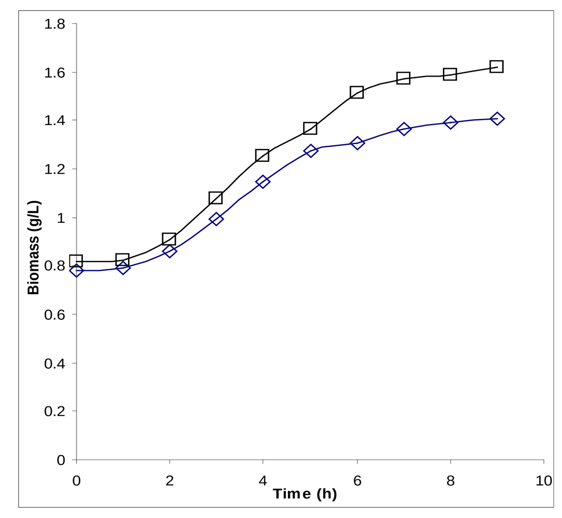
Firstly the crude extracts of the bacterial cultures were tested. Under the influence of *L.casei* treated supernatant *L.delbruckii* has a maximum growth rate of 0.16 and a doubling time of 4.31 h are achieved. The final biomass concentration is 1.01g/L. When tested against the treated supernatant of *L.plantarum L.delbruckii* has a maximum growth rate of 0.16 and a doubling time of 4.31 h are achieved. The final biomass concentration is 1.20g/L.When tested against *L.lactis* though *L.delbruckii* has a maximum growth rate of 0.14 and a doubling time of 4.92 h are achieved. The final biomass concentration is 1.24g/L. (Fig.1,2,3)



Fg.1. Growth of *L.delbruckii* (□) & l.delbruckii (◊) under L.casei supernatant

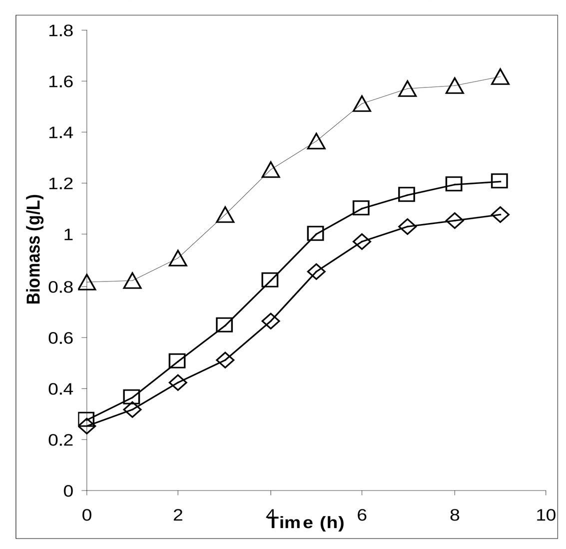


Fg.2 Growth of *L.delbruckii* (□) & l.delbruckii under L.plantarumi (◊) supernatant

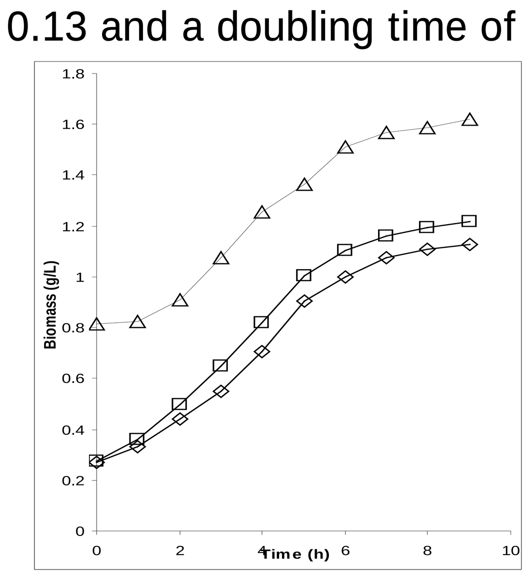


Fg.3 Growth of *L.delbruckii* (□) supernatant & l.delbruckii under L.lactis (◊)

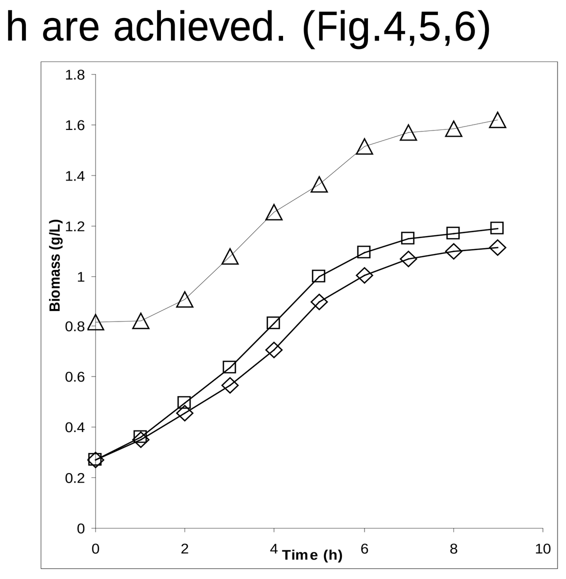
When further treated and concentrated so to avoid any interference from other antimicrobial agents existing in the supernatant the results were the following, under the influence of *L.casei* concentrated and washed supernatant *L.delbruckii* has a maximum growth rate of 0.17 and a doubling time of 4.05 h are achieved., though for the concentrated unwashed sample a maximum growth rate of 0.14 and a doubling time of 4.92 h are achieved.. When tested against the concentrated and washed supernatant of *L.plantarum L.delbruckii* has a maximum growth rate of 0.16 and a doubling time of 4.31 h are achieved. though for the concentrated unwashed sample a maximum growth rate of 0.13 and a doubling time of 5.30 h are achieved. When tested against the concentrated and washed supernatant of *L.lactis* though *L.delbruckii* has a maximum growth rate of 0.16 and a doubling time of 4.31 h are achieved, though for the concentrated unwashed sample a maximum growth rate of 0.13 and a doubling time of 5.30 h are achieved. (Fig.4,5,6)



Fg.4 Growth of *L.delbruckii* (Δ) & *l.delbruckii* under *L.casei* supernatant unwashed (◊) &washed (□) supernatant



Fg.5. Growth of *L.delbruckii* (Δ) *delbruckii* under *L.plantarumi* supernatant unwashed (◊) &washed (□) supernatant



Fg.6Growth of *L.delbruckii* (Δ) *L.delbruckii* under *L.lactis* supernatant unwashed (◊) &washed (□) supernatan

As the samples are washed all the additional antimicrobial substance existing in the supernatant are dissolved and they cannot influence *L.delbruckii* growth. On the contrary to the unwashed samples where the growth is influenced by other substances as well. According to the growth response curve fabricated *L.casei* is producing 103 IU/ml, *L.plantarum* 105 IU/ml and *L.lactis* 138 IU/ml.

Conclusions

In this work, the application of ultrafiltration to fabricate a new growth strategy to enhance bacteriocin production and growth of Lactobacilli was studied. The growth rates and doubling times of the bacilli remained high on the filtrated media and the bacteriocin production remained unaffected. Qualitative and semi quantitative experiments showed a significant potential to improve the existing growth strategy of Lactobacilli growth .Further research though has to be realised so to investigate the application of the medium into large scale.

References

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