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ORIGINAL ARTICLE

Partially chemically defined liquid medium development for intensive propagation of industrial fermentation lactobacilli strains

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Abstract An economic liquid growth medium was synthesised for high-rate production of cellular mass, lactic acid and bacteriocin in lactobacilli. Three lactobacilli that are applied extensively in industry—*Lactobacillus casei* NCIMB 11970, *Lactobacillus plantarum* NCIMB 8014, *Lactobacillus lactis* NCIMB 8586—were chosen to test the medium's efficiency. These bacteria are chemoorganotrophs requiring rich, complex media for optimum growth. Contrary to the current practice of formulating a strain-specific medium, we attempted to prepare a universal broth that would allow easy formulation and optimisation. Man de Rogosa Sharp (MRS) medium, which can support the growth of lactobacilli, was found unsuitable for use in large quantities due to its high cost of preparation and its use of beef extract and peptone from poultry as nitrogen sources, which are not environmentally friendly and have potential health risks. The developed medium supported the growth of all the three bacteria equally, offering good maximum yields and incorporating only the chemical compounds needed, resulting in an improvement in the growth rate of the bacilli of between 50 % and 241 % compared to the same strains grown on MRS. Lactic acid production was between 28.6 and 35.74 g L^{-1} and bacteriocin production ranged from 110 to 130 IU mL^{-1}.

Keywords Lactic acid bacteria · Lactobacilli · Nutrient media · MRS broth · Fermentation · Starter culture

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Introduction

Lactobacilli, due to their distinctive ability to produce organic acids from complex carbohydrates such as lactate and acetate, are applied widely in the food industry. Among the most widely employed food preservatives, the organic acids lactate and acetate suppress the pH below the growth range of most pathogenic and proteolytic bacteria, causing metabolic inhibition (Gruger and Gruger 1989). Due to the production of antimicrobial substances, namely bacteriocins, lactobacilli also help to preserve products originating from milk (Bernadeau et al. 2007). Lactobacilli are mainly responsible for the formation of the microflora of most dairy products, especially of cheese and fermented milk, being important for flavor, colour and texture. Species commonly used in dairy products include *Lactobacillus casei*, *L. helveticus*, *L. rhamnosus*, *L. lactis*, *L. curvatus* and *L. plantarum* (Jack et al. 1995). In addition to developing the quality of dairy products, lactobacilli also have beneficial effects especially on acute and chronic inflammations of the gastrointestinal tract (Frazier 1978; Ouwehand et al. 2002). Applications of lactobacilli in the food industry extend to beverages and non-beverage food products of plant origin, including grape and fruit wines, (lactobacilli malolactic fermentation) (Board 1983; Chen and Hoover 2003) fresh cabbage (sauerkraut), olives and cucumbers (pickles) (Liu 2003). Lactobacilli starter cultures provide the benefits of extended preservation, serving as a "protective shield" towards bacteriophage attack, coliform bacteria and other

pathogenic organisms (Casida 1968), while helping to maintain higher quality and safety standards as the growth of other pathogenic microorganisms is inhibited. There is a much lower probability for the production of curds to fail, thus reducing economic losses. Lactobacilli also inhibit the undesirable effects of secondary end products of bacterial metabolism, such as bitter flavours, due to alcohol production. A standard high quality of products can be achieved as the function of the starter culture is well known and its activity can be well predicted. Judicious use of starter cultures can also contribute to the development and preservation of probiotic flora, for example, the genus *Bifidobacterium*, which has been considered to be of high value in the protection of intestinal tract from various diseases, including colorectal cancer (Ayres et al. 1980)

For the successful development of starter cultures, the use of suitable nutrient media is of crucial importance, as growth media assimilate and define the nutritional conditions that determine the growth yield and metabolite productivity of the selected bacteria (Zhang and Henson 2001). Lactobacilli have complex nutritional needs, with several researchers highlighting their growth dependence on the concentration of minerals (e.g. manganese and magnesium), vitamins of the B complex, amino acids (e.g. serine), and nucleic acids (e.g. adenine) (Desjardins et al. 2001; Dembczynski and Jankowski 2002; Hoefnagel 2002; Bober and Demicri 2004). Commercially available media for lactobacilli propagation include Man De Rogosa medium (MRS), which is the most commonly used; Elliker broth; *Lactobacillus–Streptococcus* differential agar (LS agar); and All Purpose Tween agar (APT). These media are often used for research purposes and they do indeed ensure bacterial growth. However, they do not support fastidious growth, or high biomass yields due to the plethora of complex nitrogen sources, such as beef or poultry extract (peptone), they contain (Konings et al. 2000; Ostlie et al. 2003; Deegan et al. 2006; Liew et al. 2005). In addition, meat extracts cause environmental (discharged waste) and health (potential CJD or H1N1 virus) hazards, while the complexity of nutrients leads to highly expensive media formulation that is unsuitable for economically viable industrial processes for culturing lactobacilli.

For the commercialisation and mass production of lactobacilli metabolites such as bacteriocins, the modification of environmental growth factors is of major importance (Abo-Amer 2011). Bacteriocins are growth-related metabolites produced during the mid- and late-exponential growth phase, hence the vital need for the intensive propagation of lactobacilli on a well defined source. Although numerous studies have been conducted regarding the effect of physicochemical parameters from food environments on the production of bacteriocins (Delgado et al. 2005), limited research has been done on the influence of medium compounds on the production of bacteriocins. Likewise, there has been only limited study of the influence of the effect of growth conditions, both nutritional and physicochemical, on bacteriocin production (Mataragas et al. 2004; Drosinos et al. 2005; Todorov and Dicks 2005b, 2007).

Identifying the commercial opportunity of high-rate production of cellular mass of lactobacilli, an economic liquid growth medium was developed. Three industrially important lactobacilli—*Lactobacillus casei* NCIMB 11970, *Lactobacillus plantarum* NCIMB 8014 and *Lactobacillus lactis* NCIMB 8586—were chosen to test the medium's effectiveness in terms of biomass growth, lactic acid and bacteriocin production. Contrary to the current practise of formulating a strain-specific medium, a general medium was formulated successfully and optimised. The medium developed was able to support the growth of all three bacteria, offering high growth cells and products yields, and incorporating all the chemical compounds needed for growth.

Materials and methods

Materials

Yeast extract, peptone, glucose, sodium acetate, trisodium citrate, and sodium hydroxide (NaOH) were obtained from Sigma-Aldrich (Gillingham, UK).

Inoculum source

All the lactobacilli, *Lactobacillus casei* NCIMB 11970, *Lactobacillus plantarum* NCIMB 8014, and *Lactobacillus lactis* NCIMB 8586, as well as the target strain *Lactobacillus delbruckii* subsp. *lactis* NCIMB 8117 were provided in a lyophilised form by the National Collection of Industrial Food and Marine Bacteria (NCIMB, Aberdeen, UK).

Preservation of microorganisms

The bacilli were revived by inoculating the selected strains twice into 50 mL serum vials containing basal medium (MRS) and incubating statically at 30 °C (Series 6000 Incubator, Thermo Scientific, Waltham, MA) for 24 h. Stock culture solutions of each strain were prepared through a cryopreservation method (Kirsop and Snell 1984; Demain and Davies 1999). For constant use, the bacilli were regularly reinoculated (on a weekly basis) into 30 mL serum vials containing basal medium and kept at 2 °C (Kirsop and Snell 1984; Demain and Davies 1999).

Growth on basal medium: MRS broth

The specified quantities of powdered materials, namely glucose 20 g L^{-1}, yeast extract (YE) 15 g L^{-1},

bacteriological peptone (from poultry) 10 g L^{-1}, sodium acetate 5 g L^{-1}, tri-sodium citrate 2 g L^{-1}, potassium hydrogen phosphate 2 g L^{-1}, magnesium sulphate 0.2 g L^{-1} and manganese sulphate 0.05 g L^{-1} were weighed on an electronic balance (Sartorius, CP4202S, Jencons-PLS, Goettingen, Germany) and mixed in an Erlenmeyer flask containing 1 L distilled water. In order to remove the existing dissolved oxygen, the medium was boiled using a Bunsen burner. Resazurin dye functioned as an anaerobiosis indicator (negative redox potential) changing its colour from deep purple to colourless (Willis 1977). Once cooled to room temperature, the medium was dispensed into pressure tubes under the presence of gaseous nitrogen flow to achieve complete anaerobic conditions. The medium was decanted into 10 mL aliquots, which were placed into pressure tubes. The head tubes were placed under a gaseous flow of nitrogen (Gerhardt et al. 1981; Demain and Davies 1999) and then sealed with rubber stoppers and aluminium Wheaton seals. The sealed tubes were secured and autoclaved at 121 °C for 15 min. No glucose was added prior to autoclaving the medium to avoid a caramelisation effect, but glucose was then added to the required concentrations to test its effect on growth. The tubes were mixed gently on a vortex mixer, inoculated with 1 mL inoculum, and incubated statically.

Development of optimised broth medium

The effect of each major component on growth and performance of lactobacilli was tested separately using the same experimental methodology in a series of trials aimed at developing a simplified inexpensive optimised growth medium (Malek et al. 1969). The influences of carbohydrate source (glucose), nitrogen source (peptone, yeast extract), pH buffering agent (sodium acetate, tri-sodium citrate, potassium hydrogen phosphate) and metal (magnesium sulphate, manganese sulphate) were compared with MRS to investigate the optimum medium. The effect of each ingredient of the medium on the growth characteristics of the microorganisms was investigated. This was done by varying the concentration of the constituents while keeping the other components constant. Complete anaerobic conditions were achieved by dispersing the media into pressure tubes under gaseous nitrogen flow. Each major component (carbohydrates, nitrogen sources, buffering agents, metals) was prepared separately and autoclaved as a concentrated solution. The effect of each component was tested within a concentration range of 0–40 g L^{-1} and introduced to the medium via a sterile syringe in the desired amount. Each concentration was tested in triplicate to obtain average data, including positive and negative controls. After the introduction into the tubes of the desired amounts of each component, the tubes were mixed gently on a vortex mixer and then inoculated with a 1-mL inoculum. The growth rate and the amount of growth were measured spectrophotometrically. After determination of the optimum concentration for growth of each component, all components were combined resulting in the manufacture of an optimised medium (Todorov et al. 2004).

Growth on optimised broth medium and investigation of the effect of temperature on the growth of lactobacilli

After initial optimisation of the basal medium, an optimised medium with a composition of glucose 20 g L^{-1}, yeast extract 20 g L^{-1}, sodium acetate 10 g L^{-1}, tri-sodium citrate 10 g L^{-1}, potassium hydrogen phosphate 5 g L^{-1} (pH 6.2) was selected for assaying the growth and performance of the three bacteria, namely *Lactobacillus casei* NCIMB 11970, *Lactobacillus plantarum* NCIMB 8014, and *Lactobacillus lactis* NCIMB 8586. Ten pressure tubes (10 mL overall concentration) were evaluated. The contents of the tubes were mixed gently using a vortex mixer, inoculated with 1 mL inoculum, and incubated statically. The effect of incubation temperature was tested on the three strains of the *Lactobacillus* group, at 25 °C, 35 °C and 41 °C, with the optimised medium used as nutrient source (MacKane and Kandel 1996).

Measurement of cellular growth and biomass

Cellular growth was measured by placing the pressure tubes into a spectrophotometer fitted with a test tube holder with 1.8-cm light path (PU 8625 UV/VIS Philips, Suresnes, France) at 660 nm. Maximum specific growth rate (μ_{max} h^{-1}) (Bu'lock and Kristiansen 1978) and final biomass concentration (g L^{-1}) of the microbial strain were determined in basal medium under a 10 h round of static incubation at 32 °C. To convert optical density (OD) measurements into dry weight units (g L^{-1}) of bacteria, dry weight determination assays were performed (Willis 1977; Demain and Davies 1999; Avonts et al. 2004), resulting in a linear equation (two variables) with an intercept-slope form of y=mX+b for dry weight determination where X stands for OD units. The equations for *L. casei*, *L. plantarum*, *L. lactis* and *L. delbruckii* subsp. *lactis*, respectively, are the following: $Y = 0.5471\mathbf{x} + \mathbf{0.014639}$, $Y = 0.7850\mathbf{x} + \mathbf{0.17\ 09}$, $Y = 0.5755\mathbf{x} + \mathbf{0.02066}$ and $Y = 0.4266\mathbf{x} + \mathbf{0.3814}$.

Growth of lactobacilli in a pH-controlled batch reactor—effect of pH on lactic acid and bacteriocin productivity

The pH range between 4.0 and 7.0 was tested in steps of 0.5 units. A 2-L capacity pyrex glass continuously stirring tank reactor (CSTR) (Fig. 1) was used for the procedure. The round glass container had a glass cover with ports for instrumentation and sampling that were sealed with silicone rubber and fastened using metallic clamps. The fermenter was designed and equipped to achieve a constant temperature of

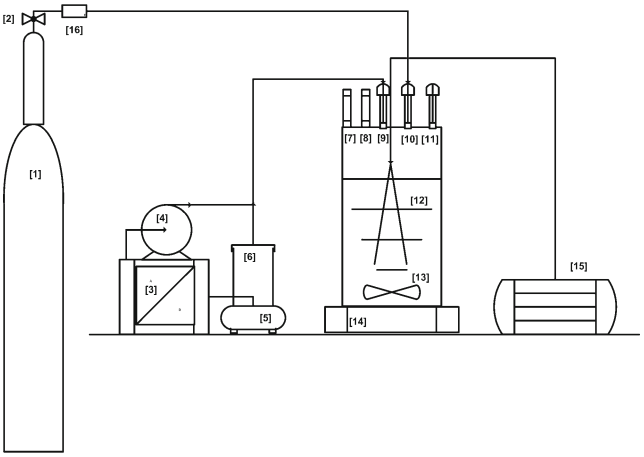

Fig. 1 Schematic representation of the continuous stirred-tank reactor (CSTR) used to study bacteriocin production from selected lactobacilli. *1* Nitrogen cylinder, *2* gas flow regulator valve, *3* pH controller apparatus, *4* centrifugal pump, *5* electronic scale, *6* alkali/acid solution vessel (1 M NaOH/HCl), *7* hydrargyric thermometer, *8* pH probe, *9* alkali/acid feed port, *10* gas exchange port equipped with a gas filter, *11* inoculation/sampling port, *12* stainless steel coils (heat emission), *13* magnetic stirrer, *14* magnetic stirring plate, *15* waterbath equipped with coils, *16* gas flow meter

35 °C and pH control with a pump to supply alkali/acid feed to the culture, as well as constant anaerobic conditions through constant flow of gaseous nitrogen through the headspace. It was equipped with a thermometer, a pH probe (Fischer Scientific, Loughborough, UK) connected to a pH controller automation apparatus (Electrolab FerMac 260, Tewkesbury, UK) that actuated a pump to supply alkali/acid feed to the culture when the level of pH was found below or above that selected, a magnetic stirrer coupled bar for agitation (350 rpm), a glass air lock, a sampling and inoculation port, a gas-in and gas-out port connected with a filter (polyvent filter, 0.2 μm, Whatman Filters, Maidstone, UK), a port for stainless steel coils, connected to a thermostatically controlled water bath, for heat transfer. Sampling was performed on an hourly basis over 10 h via the sampling port with a 10 mL sterile syringe; all samples were measured for biomass concentration, lactic acid production and bacteriocin production.

Analysis of end products using high performance liquid chromatography

Production of lactic and acetic acid by the selected lactobacilli was determined using high performance liquid chromatography (HPLC). The HPLC system was connected to a conductivity detector (ED 40 Electrochemical Detector, Dionex, Camberley, UK) and fitted with an ion exchange column (Dionex IonPac™ AS11-HC, Dionex, Oakville, ON, Canada). The mobile phase was represented by two solutions of NaOH of 0.05 mM and 100 mM concentration. The solutions were delivered to the pumps via rubber tubes and valves. The mobile phase was organised as following: NaOH 0.05 mM was poured for 5 min, then the concentration of the solution increased to 5.5 mM for 10 min and then to 100 mM for 5 min. At the final phase the NaOH concentration was kept at 0.5 mM for 15 min and then at 0.5 mM for 3 min. All samples were injected via a 20-μL injection loop. Gradient flow was applied at a flow rate of 1 mL min^{-1}, for 38 min. Temperature control of the solvents was maintained with a column incubator (Millipore, Durham , UK) at 27 °C. The operation of the system was controlled automatically using Prostar Workstation Data analysis software package (Varian, Georgetown, ON, Canada).

Bacteriocin production analysis

The activity and the potency of nisin and the produced bacteriocins were tested according to a simple turbidometric assay. This assay was based on the effect of several different concentrations of commercial nisin against a target strain, in terms of growth rate, by developing a calibration curve of different nisin concentration versus the indicator organism.

Lactobacillus delbruckii subsp. *lactis* NCIMB 8117 was selected as the target strain. The inoculum was consistent in growth phase, as it was frozen when the growth reached 1.5 g L^{-1}. The target strain was grown on a liquid medium containing 20 g L^{-1} glucose, 20 g L^{-1} YE, 10 g L^{-1} sodium acetate, 10 g L^{-1} tri-sodium citrate, 5 g L^{-1} di-hydrogen orthophosphate, magnesium sulphate 0.5 g L^{-1} and manganese sulphate 0.05 g L^{-1}. This medium was also used when testing the effect of bacteriocins and nisin. One millilitre of the frozen inoculum of *L. delbruckii* subsp. *lactis* and 1 ml of the supernatant resulting from pH control fermentations of differential concentration was added to glass tubes containing 8 mL optimised medium including metals. The samples were mixed gently, and incubated statically at 36 °C. The biomass concentration was recorded on an hourly basis by measuring the OD in a spectrophotometer (PU 8625 UV/VIS Philips) at 600 nm.

The amount of the bacteriocin produced by each strain was defined primarily in samples taken at the end of pH- and temperature-controlled fermentations. The selected samples (pH fermentation at 6.5) were transferred into 10 mL conical plastic tubes (Fisherbrand, Loughborough, UK) and centrifuged (10,000*g* 15 min) (Biofuge Stratos Sorall, Kendro Products, Langenselbold, Germany) for complete biomass removal. The clarified liquid was filtered through a 0.2 μm Whatman pore size filter for sterilisation. The pH of the sterilised liquid was adjusted at 6.0 to eliminate the antimicrobial effect of lactic acid and it was then diluted with fresh medium.

Statistical analysis

All experimental data were processed through Microsoft Excel software Version 2003 using linear regression

analysis. The data were analysed for accuracy and precision by calculating standard deviation, standard error, experimental error, regression factor and reading error. Each parameter was measured in triplicate to obtain average data (standard deviation of mean <5 %, standard error <7 %). All the numerical data were proven to be highly accurate and reproducible, having a regression factor between 0.98 and 1 and offering highly significant results.

Results and discussion

Growth performance on basal MRS medium

To evaluate μ_{max} and growth yield (X, g L^{-1}), the lactobacilli were first grown on MRS medium—the standard medium selected for lactobacilli growth (Mandolstan and MacQuillen 1973). *L. casei* had a μ_{max} of 0.16 h^{-1}. The final biomass concentration after a 10-h static incubation achieved was 1.06 g L^{-1}. *L. plantarum* had a μ_{max} of 0.12 h^{-1}. The final biomass concentration after a 10-h static incubation achieved was 1.32 g L^{-1}. *L. lactis* had a μ_{max} of 0.07 h^{-1} and the final biomass concentration after a 10-h static incubation was 0.69 g L^{-1}. The selected lactobacilli strains grew relatively slowly on MRS medium. The growth medium was therefore reformulated and simplified. Due to the materials needed, the cost of the MRS formulation was relatively high. The extensive use of nitrogen sources such as peptones from poultry or beef extract were also non-environmentally friendly, causing a high amount of waste. A step towards simplification was the omission of peptone and the evaluation of the remaining components on the growth performance of the strain. Each component was tested in a range of concentrations, while keeping all remaining parameters constant, at a concentration equal to their initial concentration in MRS medium.

Testing the parameters

Effect of nitrogen source on growth: omission of peptone

The growth results on MRS medium show that this medium is unsuitable for intensive propagation, as a medium containing numerous nitrogen sources did not facilitate good growth. The bacteriological peptone was omitted from the medium recipe and growth of lactobacilli was tested on the resulting medium. The μ_{max} of *L. casei*, *L. plantarum* and *L. lactis* were 0.22 h^{-1}, 0.18 h^{-1} and 0.19 h^{-1}, respectively, with cell concentration of 1.05 g L^{-1}, 1.00 g L^{-1} and 0.96 g L^{-1} subsequently. The absence of peptone from the medium led to an improved maximum growth rate and higher growth yields of bacteria, therefore YE was chosen as the primary and sole source of nitrogen.

Effect of YE concentration on growth

Several researchers (Sentharan et al. 1997; Todorov and Dicks 2004, 2005a, b) have introduced the idea of partial dependence of biomass development and metabolite production by lactobacilli on the amount of nitrogen sources like yeast extract within defined growth media. YE serves as the carbon, nitrogen and vitamin source needed to satisfy the growth requirements of the microorganisms. The effect of YE on growth was studied over a range of concentrations between 0 g L^{-1} and 25 g L^{-1} (Fig. 2a) with μ_{max} of the bacteria increasing simultaneously with the concentration of the component. For *L. casei*, *L. plantarum* and *L. lactis*, the highest μ_{max} values of 0.21 h^{-1}, 0.29 h^{-1} at 0.30 h^{-1} with biomass concentrations of 1.00 g L^{-1}, 1.60 g L^{-1} and 1.03 g L^{-1}, were achieved at a concentration of 20 g L^{-1} YE. Further increase in biomass was not observed, most probably due to exhaustion of the carbohydrate source.

Effect of metal ion concentrations on growth

Several studies have underlined the important influence of metal ions in providing cations for metabolism in the growth of lactobacilli (Carr et al. 1975). The effects of manganese and magnesium salts on the growth of *L. casei*, *L. plantarum* and *L. casei* were studied over a range of concentrations between 0 g L^{-1} and 1.5 g L^{-1}.

For *L. casei*, the highest μ_{max} rate for magnesium sulphate was 0.17 h^{-1} at 0.0 g L^{-1}, when no magnesium sulphate was added. For manganese sulphate, the highest μ_{max} rate was 0.23 h^{-1}, when no manganese sulphate was added. A similar pattern was followed by *L. plantarum*, yielding 0.30 $h^{-1}$and 0.24 h^{-1}, respectively, in the absence of metal ions. *L. lactis* also grows better in the absence of magnesium and manganese sulphate, with a μ_{max} of 0.30 h^{-1} and 0.20 h^{-1}. The growth of lactobacilli is strongly influenced by concentrations of metal ions higher than 2.5 g L^{-1}, suggesting that the rich nitrogen source of YE might contain enough ions to support growth (Fig. 3).

Effect of glucose concentration on growth

As the yield of biomass in all anaerobic bacteria is strongly dependent on carbohydrate feed (Todorov and Dicks 2004), glucose was used as an energy source. The effect of glucose concentration was studied in a range of concentrations between 0 g L^{-1} and 40 g L^{-1} (277 mM) (Fig. 2b). Growth of the tested strains, in terms of both yield and growth rate, was stimulated by the addition of glucose up to a

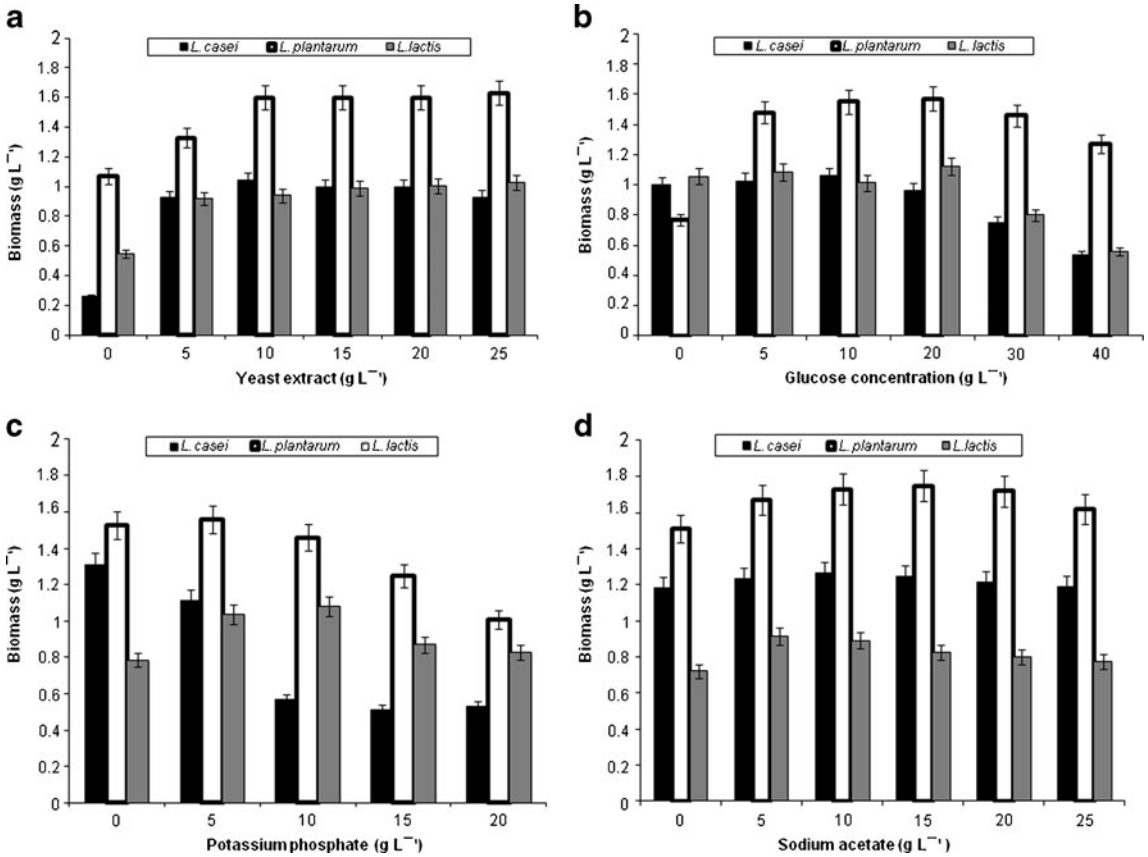

Fig. 2a–d Effect of different nutrient concentrations on lactobacilli growth. **a** Yeast extract, **b** glucose, **c** potassium phosphate, **d** sodium acetate concentration

concentration of 20 g L^{-1}; above 20 g L^{-1} the organisms' growth was inhibited.

Lactobacillus casei was not greatly affected by the rise in glucose concentration, with μ_{max} and biomass both rising simultaneously with increasing glucose concentration, although 40 g L^{-1} became slightly inhibitory for growth. The optimum concentration of glucose was found to be 20 g L^{-1} (111 mM) (μ_{max} 0.24 h^{-1}). *L.*

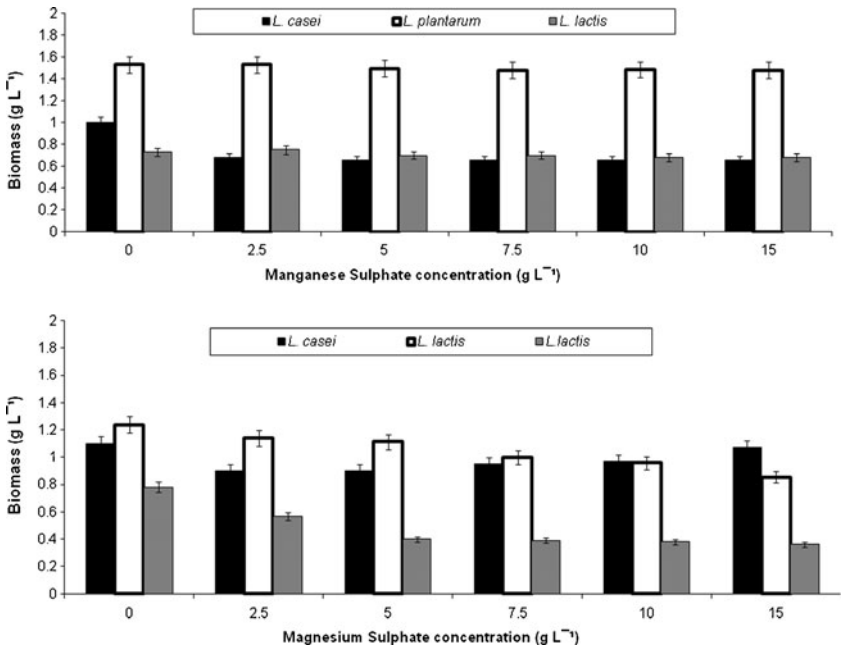

Fig. 3 Effect of different metal ion concentrations on growth of lactobacilli

plantarum followed a similar pattern but glucose became strongly inhibitory for growth at 30 g L^{-1} (167 mM). The highest μ_{max} (0.26 h^{-1}) was observed at 20 g L^{-1}. *L. lactis* proved to be sensitive to alterations in glucose concentrations, with 30 g L^{-1} (167 mM) glucose being growth inhibitory. A concentration of 20 g L^{-1} (μ_{max} 0.24 h^{-1}) was selected as the optimum.

Glucose is a carbon as well as an energy source for microbial growth, and has been correlated mainly with production of cellular biomass, while in the case of lactic acid bacteria (LAB), glucose has been considered to be converted to lactic or acetic acid, i.e. carbon for structure, depending on the bacterial strain used as well as the medium composition and physicochemical culturing conditions (Hofvendahl et al. 1999; Hofvendahl and Hahn-Hägerdal 2000). Numerous studies on LAB growth and acid production have been conducted using other carbohydrate sources such as fructose, mannose, lactose, xylose and starch (Jung and Lovitt 2010a, b; Jung et al. 2011) in an effort to assimilate the growth of strains on natural resources rich in sugars or to examine the metabolic pathways used by bacteria and to correlate their effect with lactic or acetic acid production.

However, for numerous strains of LAB such as *Leuconostoc mesenteroides* E131, biomass production on lactose, fructose and sucrose has never exceeded 1.25 g L^{-1} (Metsoviti et al. 2011) while with glucose at 20 g L^{-1} biomass can reach 2.16 g L^{-1} (Drosinos et al. 2005, 2006). Higher concentrations of glucose, in some cases, do provide higher cellular growth (Aktypis et al. 2007); in the case of lactobacilli they were considered inhibitory for growth.

Effect of potassium hydrogen phosphate on growth

The effects of potassium hydrogen phosphate—the buffering component of the medium—on the growth of the selected lactobacilli were studied. The effect of addition of phosphate was in a range of concentrations between 0 g L^{-1} and 20 g L^{-1} (Fig. 2c). Growth was inhibited by high potassium hydrogen phosphate concentrations, although this component cannot be omitted completely, as indicated by the increase in growth upon small additions of phosphate. A concentration of 5 g L^{-1} was selected as the optimum, giving μ_{max} values for *L. casei*, *L. plantarum* and *L. lactis* of 0.21 h^{-1}, 0.21 h^{-1} and 0.19 h^{-1}, respectively, with cell concentrations of 1.11 g L^{-1}, 1.56 g L^{-1} and 1.04 g L^{-1} subsequently.

Effect of sodium acetate on growth

The effect of sodium acetate, which acts as a buffer and selective agent causing inhibition of growth of bacteria of other species as well as a carbon source, was studied over a range of concentrations between 0 g L^{-1} and 25 g L^{-1} (Fig. 2d). The organisms were not subsequently affected by high sodium acetate concentrations at pH 6.5, suggesting that the selected lactobacilli are acetic acid tolerant and do not produce a high amount of acetic acid as an end product of their metabolism.

The μ_{max} values of *L. casei*, *L. plantarum* and *L. lactis* were at 10 g L^{-1} were 0.20 h^{-1}, 0.20 h^{-1} and 0.15 h^{-1}, respectively, with cell concentrations of 1.25 g L^{-1}, 1.73 g L^{-1} and 0.89 g L^{-1} subsequently.

Effect of tri-sodium citrate on growth

Tri-sodium citrate—a component acting both as a selective buffer and carbon source and a specialised energy source (as the starting point of the tricarboxylic acid cycle, citrate is a key electron acceptor and metabolic intermediate)—was studied over a range of concentrations between 0 g L^{-1} and 25 g L^{-1}. The μ_{max} values of the bacilli were enhanced by tri-sodium citrate up to a concentration of 15 g L^{-1}. The μ_{max} of *L. casei*, *L. plantarum* and *L. lactis* were 0.28 h^{-1}, 0.30 h^{-1} and 0.22 h^{-1}, respectively, with cell concentrations of 1.26 g L^{-1}, 1.63 g L^{-1} and 1.31 g L^{-1} subsequently; 10 g L^{-1} was chosen as the optimum value.

Growth on optimum medium

Combining all the optimised growth parameters in the desired quantities formulated a liquid medium. This developed medium served the aim of enhancing cellular productivity, ensuring high growth. Comparing growth of the selected lactobacilli on MRS and the formulated medium (Table 1) clearly demonstrated that the optimised medium improves growth with a significant high increase in the maximum specific growth rate and on the final cell concentration. The μ_{max} of *L. casei*, *L. plantarum* and *L. lactis* were 0.24 h^{-1}, 0.30 h^{-1} and 0.22 h^{-1}, respectively, with cell concentrations of 2.43 g L^{-1}, 2.63 g L^{-1} and 1.81 g L^{-1}, respectively. The optimised medium was then used for further investigative studies. Further testing had to be done to achieve suitable physicochemical conditions such as temperature and pH.

Effect of physical conditions, temperature and pH on growth of lactobacilli

To further optimise growth rate and biomass concentration, the effect of temperature on growth of the lactobacilli was tested in test tubes containing the formulated medium. The optimum temperature selected was used throughout the overall experimental study as the ideal temperature to ensure sufficient growth. Several researchers (Holt 1974; Sentharan et al. 1997; Todorov and Dicks 2004, 2005a, b) proposed an incubation temperature for lactobacilli in the range of 25–38 °C. The temperatures selected for this study were 25 °C,

Table 1 Comparison of the effect on lactobacilli growth of basal (MRS) and optimised medium

Lactobacillus	Medium	μ_{max}, h^{-1}	μ_{max}, h^{-1} optimisation percentage (%)	Total glucose consumption (g L^{-1})	Final biomass concentration (g L^{-1})	Final biomass concentration optimisation percentage (%)
L. casei	Basal	0.16	50	11.82	1.19	104
	Optimised	0.24		19.22	2.43	
L. plantarum	Basal	0.13	130	11.46	1.32	99
	Optimised	0.30		19.86	2.63	
L. lactis	Basal	0.07	214	8.54	0.69	162
	Optimised	0.22		19.00	1.81	

35 °C and 41 °C. At 35 °C,*L. casei* had a growth rate of 0.26 h^{-1}, while at 25 °C and 41 °C the growth rate was 0.07 h^{-1} and 0.13 h^{-1}, respectively. At 35 °C, *L. plantarum* had a growth rate of 0.31 h^{-1}, and at 25 °C and 41 °C the growth rates were 0.07 h^{-1} and 0.12 h^{-1}, respectively. *L. lactis* at 35 °C had a growth rate of 0.25 h^{-1}, although at 25 °C and 41 °C the growth rate was 0.06 h^{-1} and 0.03 h^{-1}, respectively. The strains proved to be influenced strongly by oscillation of the temperature of incubation as they did not grow at temperatures below 30 °C and above 35 °C (Fig. 4). As such, an optimum temperature of 35 °C was selected for all the lactobacilli tested.

The maximum specific growth rate of all three lactobacilli was enhanced when the bacteria were cultured at a controlled pH of 6.5, though in *L. lactis* and *L. plantarum* pH 7 also supported good growth. These experiments gave higher biomass yields and maximum specific growth rates compared to uncontrolled pH growth systems. The effect of pH was tested in a 2-L CSTR operated batchwise, using the formulated medium as substrate. It was also observed that, at acidic pH values of 4 and 4.5, growth of the bacilli was inhibited completely. In this experimental set up, the amount of organic acid (mainly lactic acid) produced by the bacilli is equal to the amount of NaOH used for pH maintenance. Over the 10-h fermentation at pH 5, 5.5 and 6, the organisms were still growing (Fig. 5) although they had slower maximum specific growth rates and long lag periods prior to growth.

For *L. casei* the highest μ_{max} was between 0.19 h^{-1} and 0.21 h^{-1} at pH 6.0 and 6.5, respectively. There was no significant change compared to the uncontrolled pH system, and the cellular yield remained the same. The performance of the uncontrolled system was close to that at pH 6.5 in this system, which is selected as the optimum. For *L. plantarum*, the highest μ_{max} was between 0.22 h^{-1} and 0.30 h^{-1} at pH 6.5 and 7.0, respectively. The performance of the uncontrolled system was close to that at pH 6.5 in this system, which was selected as the optimum. It was clear that, although *L. plantarum* growth was quite sensitive to alkali conditions as well as to extreme acidic points, while the other two strains were far more acid tolerant. For *L. lactis* the highest μ_{max} values were between 0.15 h^{-1} and 0.19 h^{-1} at pH 7.0 and 6.5, respectively, and the cellular yield became slightly higher. The performance of the uncontrolled system was close to that at pH 6.5 in this system, which was selected as the optimum. However, it was clear that growth of *L. lactis* was also quite sensitive to alkali as well as to extreme acidic points.

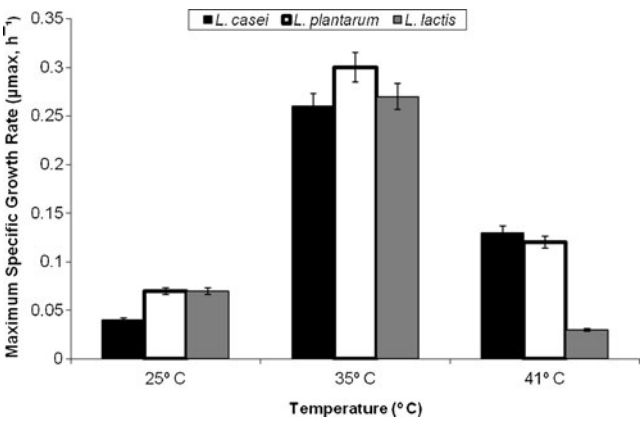

Fig. 4 Effect of different temperatures on lactobacilli growth

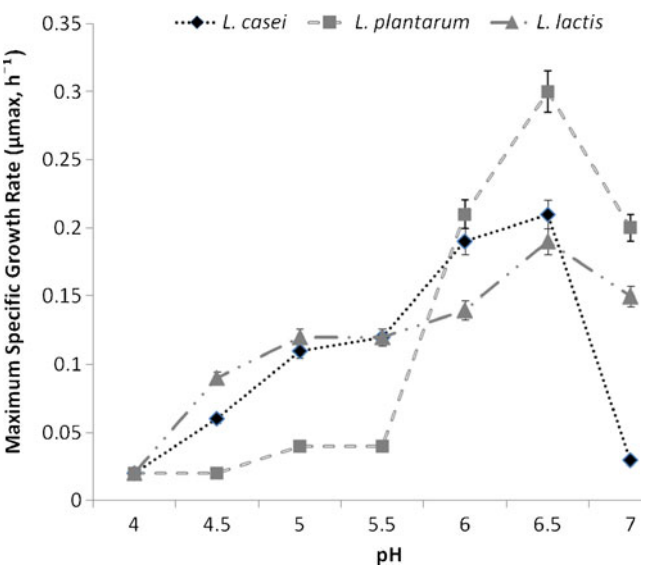

Fig. 5 Effect of different pH values on growth of lactobacilli

Determination of lactic acid and bacteriocin production

A higher amount of lactic acid production was observed at pH 6.5 for the selected lactobacilli. As the selected lactobacilli are homolactic it is assumed that the glucose utilised for growth is directly converted to lactic acid. During the exponential growth phase, all three bacilli produced high amounts of lactic acid when grown on the optimised medium. The production of lactic acid and bacteriocin from the three lactobacilli was investigated in a 2-L capacity batchwise-operated CSTR, using the optimised formulated medium. Samples for biomass, lactic acid and bacteriocin determination were harvested on an hourly basis. For *L. casei*, 30.75 g L^{-1} lactic acid was produced, *L. plantarum* produced 35.74 g $L^{-1}$and *L. lactis* 28.6 g L^{-1}. Similar results in lactic acid production by these organisms have been found in the literature (Hujanen and Linko 1996; Fu and Mathews 1999; Wee et al. 2006); however, these are related to growth of the bacteria on lactose-based media and food sources, making a strong case for use of the developed medium as an alternative solution.

The suitability of the developed medium for bacteriocin production was also tested. The bacilli were found to produce significant amount of bacteriocins (*L. casei* 110 IU mL^{-1}, *L. plantarum* 125 IU mL^{-1} and *L. lactis* 115 IU mL^{-1}).

Several studies have measured the effect of culturing conditions on the production of bacteriocins by LAB (Drosinos et al. 2005, 2006, Aktypis et al. 2007). Bacteriocins behave as primary metabolites, with their production correlating strongly with biomass growth. It has been noted, however, that temperature and pH growth-related optimum values are not always optimum for bacteriocin production, especially in the case of *Leuconostoc mesenteroides* (Drosinos et al. 2005, 2006; Aktypis et al. 2007). For lactococcal pediococcal and lactobacilli bacteriocins, production is influenced positively when maximum growth rates are maintained (Drosinos et al. 2005; Todorov and Dicks 2006).

Bacteriocin production has been tested widely in various commercial media as well as food sources (Todorov and Dicks 2005b, 2007; Metsoviti et al. 2011). Interestingly, several researchers have suggested production on low value raw materials, residues or by-products (Todorov and Dicks 2005b, 2007; Metsoviti et al. 2011). The benefits of this approach would include minimisation of environmental impact due to the re-use of waste sources, which are available in abundance, and ensuring minimising of production costs, while valorising waste sources. It has also been reported (Metsoviti et al. 2011) that production of bacteriocin by LAB propagated on sugar refining wastes (molasses) also led to the decolourisation of these sources, suggesting the development of a novel environmentally friendly treatment process for these wastes with simultaneous production of valuable substances such as bacteriocins.

Bacteriocin detection assays

The lack of a commonly accepted assay for bacteriocin quantification precludes a detailed comparison between the available literature data.

Inhibition of growth can be detected using numerous assays, such as direct microscopic counts or measuring absorbance (Delgado et al. 2005); assays can also incorporate a fluorimetric or colorimetric growth indicator (Budde and Rasch 2001), turbidity (Kawai et al. 1997), dry weight and zones of inhibition (Patton et al. 2006). Other alternatives that have been proposed include methods based on flow cytometry, conductance (Giraffa et al. 1990), measurement of ATP, bioluminesce (Simon et al. 2001) and immunological methods (competitive direct and indirect ELISA) (Daoudi et al. 2001). These techniques, despite their alleged high sensitivity, have not yet received wide acceptance for bacteriocin quantification because of the requirement of highly qualified, trained operators, dedicated equipment and expensive supplies (Delgado et al. 2005).

Thus, the agar plate diffusion assay (ADA) (Mocquot and Lefebvre 1956; Tramer and Fowler 1964) and its variations (agar well diffusion assay, agar spot test) are currently the preferred method for bacteriocin activity quantification.

In spite of its simplicity, the ADA method has numerous limitations, such as the limited diffusivity of bacteriocins in the agar medium due to their hydrophobicity (Guillaume et al. 2004); poor accuracy, especially on bacteriocin production studies (Turcottea et al. 2004); and subjective interpretations of the unit of bacteriocin defined as the minimum concentration of the compound that produces a “clear” inhibition zone (Guillaume et al. 2004). Results rely almost completely on human ability and judgment, and the required precision cannot by acquired if the inhibition zone is unclear (not perfectly circular) (Vesterlund et al. 2004). It is also a time-consuming, arduous method, that requires preparation and cooling of Petri dishes, boring of test wells in the agar and manual measurement of inhibition zones after a certain period of time (Parente et al. 1996).

To improve quantification and sensitivity in the testing of antimicrobial activity of bacteriocins in crude form, a quick, accurate, easy to implement, simple and cost effective method was developed, based on the idea of correlating bacterial growth, of the target strain in terms of μ_{max}, with the inhibitory effect of the only commercially available lactobacilli bacteriocin nisin.

It can be concluded easily from the experimental results presented here that MRS medium, although it can support the growth of lactobacilli, is unsuitable for use in large quantities due to its high cost of formulation and its content of nitrogen sources of beef extract and peptone from poultry, which are not environmentally friendly due to potential hazards.

To address these hurdles, a simplified, cost-effective medium has been developed and tested for lactobacilli growth. The effectiveness of the medium was tested combined with the use of the optimum pH values resulting in an improvement of the growth rate of the strains of between 50 % and 241 %. The optimised medium was proven to be highly effective for biomass production as well as bacteriocin production.

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