Investigation of Shelf life of Potency and Activity of the *Lactobacilli* produced Bacteriocins through their exposure to various physicochemical stress factors

Dr. Myrto-Panagiota Zacharof ¹

Dr. Robert W. Lovitt ²

¹ *Multidisciplinary Nanotechnology Centre,
Swansea University, Swansea, SA2 8PP, UK*

² *College of Engineering, Multidisciplinary
Nanotechnology Centre, Swansea University,
Swansea, SA2 8PP, UK*

Swansea University
Prifysgol Abertawe

Project Summary

- Optimisation of physicochemical conditions of in vitro media for growth and bacteriocin production from *Lactobacilli*
- Development of a dose-response model for quantifying the bacteriocin by *Lactobacilli* amount produced based on the effect of Nisin (LAB commercially available bacteriocin) on the target strain
- Development of an extraction-concentration strategy of the bacteriocins majorly based on membrane filtration.

Project Summary

- Testing the Activity and the Potency of the Commercially Available Nisin and the Produced Bacteriocins with Heat Treatment
- Testing the Activity and the Potency of the Commercially Available Nisin and the Produced Bacteriocins Through Storage effect on low temperatures
- Testing the Activity and the Potency of the Commercially Available Nisin and the Produced Bacteriocins through Dilution

Introduction

- *Lactobacilli*: Rod shaped bacteria widely used in the food industry nowadays.
- They are mostly used in dairy industry due to their ability to ferment sugars especially glucose and galactose so to produce lactic acid .
- Research suggests that they produce antimicrobial activity metabolites
- These are active against other bacteria of the same genre and could be used as natural antimicrobials on their own or synergistically with other factors.

Introduction

- Their production mechanism has been widely studied as well as their antimicrobial activity spectrum.
- Their antimicrobial nature is rather bacteriostatic than bactericidal as they mostly affect the cellular membrane of the target strains inactivating their reproduction mechanism.
- Nisin is the only commercially available bacteriocin. This emerges the need to further exploit these metabolites.
- The use of nisin as a natural additive to food, has been approved worldwide.

Introduction

- Their production has several implications :
- They are extracellular metabolites of peptide nature so they are sensitive to conditions that may denaturise their peptide nature.
- If produced in large scale, using traditional fermentation techniques it might not be feasible to be extracted immediately from the fermented broths. Factors such as mechanical stress, dilution rate of the nutrient media, agitation or aeration might affect the bacteriocins quality.
- They were growth related metabolites so they are influenced from factors that negatively affect microbial growth (end product inhibition due to lactate excess, glucose exhaustion due to fastidious growth, microbial contamination).
- Their extraction becomes difficult as they may form micelles or clumps with the nitrogen sources already in the production medium.

Aims and Objectives

- In this project, the previously referred problems were taken into consideration and a suitable strategy was developed.
- Three known bacteriocin producing strains were selected.
- A common optimised growth medium was be developed including optimum physicochemical conditions.

Aims and Objectives

- The quantification method included a carefully selected target strain and a growth medium that ensured optimum growth of the target strain was developed.
- The self life of potency and activity was evaluated by exposing the extracted-concentrated bacteriocins and the crude extracts into several physicochemical stress factors.

Materials and Methods

- Chemicals supplied by Sigma Aldrich UK, Fischer UK, Fluka UK.
- *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), Ferguson Building, Craibstone Estate, Aberdeen , Scotland, AB21 QYA.
- Pyrex glass tubes sealed with butyl rubber stoppers and alumina seals were used to measure the growth rate. The microbial growth was measured on UV/Vis Spectrometer Philips 8250 and the numerical results are calculated on MS Excel software 2003.

Fig 1. Test tubes containing growth medium

Fig 2. UV/Vis Philips 8250 Spectrophotometer

Materials and Methods

- The amount of bacteriocins produced was measured by analysing the protein content using the Lowry method.
- Bacteriocins were sized using Dynamic Light Scattering technology.
- In order to quantify the amount of bacteriocins produced a liquid turbidometric assay based on nisin was developed
- Their activity and potency was tested against the target strain *L.delbruckii*
- To concentrate and purify the produced bacteriocins a serie of membrane filters of 30 kDa (Cellulose acetate) , 4kDa and 1kDa (Polysulfone) MWCO were used. The filters were purchased by Millipore, Microdyn-Nadir and Osmonics and fitted in Amicon cell 8200 unit reactor

Fig 3. Amicon Cell Unit Reactor 8200 System

Fig 4. Ultrafiltration membrane filters (30kDa, 4kDa, 1kDa counter clockwise)

Results

Growth Media Development and Optimisation

1.	Glucose ($C_6H_{12}O_6$)	2% w/v
2.	Yeast Extract (Y.E.)	1.5% w/v
3.	Bacteriological Peptone	1% w/v
4.	Sodium acetate (CH_3COONa)	0.5% w/v
5.	Tri-Sodium citrate($C_6H_5Na_3O_7$)	0.2% w/v
6.	Potassium Hydrogen Phosphate (KH_2PO_4)	0.2% w/v
7.	Magnesium Sulphate ($MgSO_4 \cdot 7H_2O$)	0.02% w/v
8.	Manganese Sulphate ($MnSO_4 \cdot 3H_2O$)	0.005% w/v
9.	Resazurin Dye ($C_{12}H_4N_4O_6$)	0.005% v/v

Table 1: MRS Modified Medium (Basal Medium for growth)

1.	Glucose ($C_6H_{12}O_6$)	2% w/v
2.	Yeast Extract(Y.E.)	2% w/v
3.	Sodium acetate (CH_3COONa)	1% w/v
4.	Tri-Sodium citrate ($C_6H_5Na_3O_7$)	1% w/v
5.	Potassium Hydrogen Phosphate ($KHPQ$)	0.5% w/v
6.	Resazurin Dye ($C_{12}H_4N_4O_6$)	0.005% v/v

Table 2: Optimised Medium for Growth

Results

Growth Media Development and Optimisation

Bacteria	Media	Maximum specific Growth rate ($\mu_{\max}$, h ⁻¹)	Doubling time (td, h)	Final Biomass (g/L)
<i>L.casei</i>	<i>MRS modified medium</i>	0.16	4.31	1.19
	<i>Optimised Medium</i>	0.24	2.87	2.43
<i>L.plantarum</i>	<i>MRS modified medium</i>	0.13	5.30	1.32
	<i>Optimised Medium</i>	0.30	2.30	2.63
<i>L.lactis</i>	<i>MRS modified medium</i>	0.07	9.85	0.69
	<i>Optimised Medium</i>	0.22	3.13	1.81

Table 3: Comparative table of performance Optimised Medium and MRS modified medium

Results

Development of a Quantification Assay of the Produced Bacteriocins

Fig 5. Standard curve of quantification of nisin assay

- The assay was developed based on the growth rates of the target strain *L. delbruckii* occurring under different concentrations of nisin induced in the medium.
- The assay had a limit value of 200 IU/ml of nisin or of nisin-like activity antimicrobial compound. Beyond this value no growth occurs.
- The *Lactobacilli* were tested for bacteriocin production both through measurement of protein content and through testing the supernatant for antimicrobial activity.

Results

Bacteriocin production by the selected *Lactobacilli* Crude extracts and concentrated bacteriocins

<i>Lactobacilli</i>	Growth rate (μ , h ⁻¹)	Doubling Time (td,h)	Amount of bacteriocins produced (IU/ml)
<i>L.casei</i>	0.16	4.31	95
<i>L.plantarum</i>	0.16	4.31	95
<i>L.lactis</i>	0.14	4.92	105

**Table 4. Bacteriocin from *Lactobacilli*
crude extracts**

<i>Lactobacilli</i>	Growth rate (μ , h ⁻¹)	Doubling Time (td,h)	Amount of bacteriocins produced (IU/ml)
<i>L.casei</i>	0.12	5.75	115
<i>L.plantarum</i>	0.10	6.90	125
<i>L.lactis</i>	0.09	7.66	130

**Table 5. Concentrated (1kDa MWCO)
Bacteriocins from *Lactobacilli***

Results

Potency of Bacteriocins and its Resistance to Deactivating Factors

Figure 6 Effect of relative concentration dilution on the potency of nisin solution 1000 IU/ml

Results

Potency of Bacteriocins and its Resistance to Deactivating Factors

Fig 7. Potency of *L.casei* (◇) *L.plantarum* (□) & *L.lactis* (Δ) produced Bacteriocin on Optimised Media and relative concentration dilution

Results

Potency of Bacteriocins and its Resistance to Deactivating Factors

Figure 9 Stability of potency of *L. casei* bacteriocin up to 96h treated with heat (80 °C) (◇) treated with microfiltration (□) & without any treatment (Δ)

Figure 10 Stability of potency of *L. plantartum* bacteriocin up to 96h treated with heat (80 °C) (◇) treated with microfiltration (□) & without any treatment (Δ)

Figure 11 Stability of potency of *L. lactis* bacteriocin up to 96h treated with heat (80 °C) (◇) treated with microfiltration (□) & without any treatment (Δ)

The potency of the bacteriocins was tested under numerous conditions that could possibly deactivate them.

These conditions include heat treatment, storage capacity, filtration and dilution.

It was proven that bacteriocins are relatively stable molecules that should be commercially exploited.

Results

Stability of the Bacteriocins produced from the selected Lactobacilli *L.delbruckii*

<i>Lactobacilli</i> <i>Bacteriocins</i>	<i>Concentrated extracts</i>								
	24h			48 h			72 h		
	<i>Growth rate</i> <i>(μ, h-1)</i>	<i>Doubling time</i> <i>(td)</i>	<i>Potency</i> <i>IU/ml</i>	<i>Growth rate</i> <i>(μ, h-1)</i>	<i>Doubling time (td)</i>	<i>Potency</i> <i>IU/ml</i>	<i>Growth rate</i> <i>(μ, h-1)</i>	<i>Doubling time (td)</i>	<i>Potency</i> <i>IU/ml</i>
<i>L.casei</i>	0.15	4.60	100	0.001	Stationary phase	100	-0.003	No growth	100
<i>L.plantarum</i>	0.16	4.31	95	0.007	Stationary phase	95	-0.002	No growth	95
<i>L.lactis</i>	0.14	4.92	103	0.006	Stationary phase	103	-0.001	No growth	103

Table 6. Stability of Bacteriocin from *Lactobacilli* crude extracts

Results

Stability of the Bacteriocins produced from the selected *Lactobacilli* *L.delbruckii*

<i>Lactobacilli</i> <i>Bacteriocins</i>	<i>Concentrated extracts</i>						
	<i>24h</i>			<i>48 h</i>			
	<i>Growth rate</i> (μ , h-1)	<i>Doubling time</i> (td)	<i>Potency</i> IU/ml	<i>Growth rate</i> (μ , h-1)	<i>Doubling time</i> (td)	<i>Potency</i> IU/ml	
<i>L.casei</i>	0.07	9.85	150	-0.006	No growth	150	
<i>L.plantarum</i>	0.06	11.5	155	-0.003	No growth	155	
<i>L.lactis</i>	0.06	11.5	155	-0.002	No growth	155	

Table 7. Stability of Concentrated Bacteriocin from *Lactobacilli* crude extracts

Conclusions and Future Recommendations

Conclusions

- An optimised growth medium for *Lactobacilli* and the in vitro production of bacteriocins was developed.
- A novel quantification method of their amount was developed.
- Bacteriocins are proven to be thermostable, resistant to mechanical stress and with strong potency.

Future work

- A mathematical model could be developed forecasting the production of bacteriocins.
- The bacteriocins could be tested in a wider area of target strains.
- Several other nutrient sources could be tested.

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Questions

Email:

M.Zacharof@swansea.ac.uk

Thank you!!!