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Farzana Beegum MA

M tech scholar, Department of Dairy Microbiology College of Dairy Science and Technology, Kerala Veterinary and Animal Sciences University, Trissur, Kerala, India

Ligimol James

Assistant Professor, Department of Dairy Microbiology College of Dairy Science and Technology, Kerala Veterinary and Animal Sciences University, Trissur, Kerala, India

Beena AK

Professor and Head, Department of Dairy Microbiology College of Dairy Science and Technology, Kerala Veterinary and Animal Sciences University, Trissur, Kerala, India

Aparna SV

Assistant Professor, Department of Dairy Microbiology College of Dairy Science and Technology, Kerala Veterinary and Animal Sciences University, Trissur, Kerala, India

Aysha CH

Assistant Professor, Department of Food Microbiology College of Food Technology, Kerala Veterinary and Animal Sciences University, Trissur, Kerala, India

Corresponding Author:

Farzana Beegum MA

M tech scholar, Department of Dairy Microbiology College of Dairy Science and Technology, Kerala Veterinary and Animal Sciences University, Trissur, Kerala, India

Inhibitory effect of metabolites of *Lactobacillus fermentum* MH782089 grown in sucrose and dipotassium hydrogen phosphate supplemented paneer whey against *Staphylococcus aureus*

Farzana Beegum MA, Ligimol James, Beena AK, Aparna SV and Aysha CH

Abstract

Study aimed at assessing suitability of paneer whey as growth medium for production of antimicrobial metabolites by *Lb. fermentum* MH 782089 against *S. aureus*. Smaller zone of clearance (ZOC) was exhibited by sterilized paneer whey cell free supernatant (PW-CFS) compared to the control MRS broth cell free supernatant (MRS broth- CFS) when incubated at 37°C for 48h. As antimicrobial activity exhibited by PW-CFS was lower than MRS broth-CFS attempts were made to increase efficiency of PW as growth medium by supplementing it with different levels of sucrose and dipotassium hydrogen phosphate. PW supplemented with 3% sucrose and 0.3% buffering agent (sPW) exhibited maximum ZOC (22.6±0.5). Production conditions were optimised in terms of incubation temperature and period. Highest antimicrobial activity was exhibited in sPW at 37 °C for 72h. Thus sterilized paneer whey can be developed as an alternative for MRS broth as growth medium for the production of antimicrobial metabolites by *Lb. fermentum* MH 782089.

Keywords: *Lb. fermentum*, antimicrobial activity, supplemented paneer whey cell free supernatant

Introduction

Whey is the watery part of milk remaining after the separation of curd obtained upon coagulation of milk protein by acid or proteolytic enzymes (Panesar *et al.*, 2007) [8]. The whey thus obtained as the by-product during manufacture of paneer, cheese etc. is highly nutritious and is recognized as a potent pollutant with major disposal problems. Many options are being attempted for addressing this issue but without much result. As this by product is a rich source of many nutrients it could serve as a potential option for use as a growth medium for cultivation of microorganisms especially lactic acid bacteria. Such an initiative can result in a drastic impact on the economics of growth formulations for lactic acid bacteria (LAB) considering their highly fastidious nature and the necessity of mass cultivation for many of its industrial applications. Additionally it will address the pollution and disposal problems associated with whey in a highly environment friendly manner. Among LAB, members of genus *Lactobacillus* are widely used for food fermentations. Fermented foods containing lactobacilli include many ancient staples, ranging from fermented milk, yogurt, cheese and wine to olives, pickles, sauerkraut, sourdough bread, fermented sausages and salami, as well as silage and recent probiotic dairy products (Bernardeau *et al.*, 2006). Among lactobacilli, the species *Lactobacillus fermentum* is recognized as an economically important species due to its wide use in the production and preservation of fermented food as an acid producing starter culture (Swain *et al.*, 2014). *Lactobacillus fermentum*, an obligately hetero fermentative lactobacilli species originate from variety of habitats: traditionally fermented milk products, sourdough, fermenting plant materials, faeces and sewage amongst others as reviewed by Dan *et al.*, 2015 [4]. It is also recognized as a member of the non-starter LAB population in some cheese varieties. Many strains of *L fermentum* are reported to be of industrial relevance in terms of their ability to produce antimicrobial agents, enzymes and as a starter culture for traditionally fermented products (Song *et al.*, 2014). *Lactobacillus fermentum* MH782089 used in this study is an isolate from orange and was found to have good antagonistic potential when grown in MRS broth. Considering the economic significance of developing a low cost medium

for the cultivation of this putative antagonistic strain an attempt was made to explore the possibilities of using paneer whey as the growth medium for the cultivation of this organism.

Materials and Methods

Microbial cultures

Lactobacillus fermentum MH782089 used in this study was isolated from orange. The indicator organism used was *Staphylococcus aureus*, an isolate from milk maintained as glycerol stock in the Culture collection of the Department of Dairy Microbiology. Both the cultures were stored under refrigeration condition and whenever required the cultures were activated by inoculating in sterilized media and subsequent incubation.

Suitability of sterile paneer whey as growth medium for antimicrobial metabolite production

Suitability of sterile paneer whey as a growth medium for the production of antimicrobial agents was assessed by checking the antimicrobial activity of the paneer whey cell free supernatant (PW-CFS) against *Staphylococcus aureus* by agar well diffusion bioassay as per Herreros *et al.* (2005) [6] keeping MRS broth- CFS (MRS-CFS) as control. For this overnight incubated culture of indicator organism was adjusted to an optical density (OD) of 0.3 (which corresponds to 107 to 108 CFU/ml cells,) at 540nm and was spread on pre-set Mueller Hinton agar (MHA, HiMedia Laboratories Pvt. Ltd., Mumbai) plates. The CFS was prepared by centrifugation of overnight incubated, *Lactobacillus fermentum* MH782089, inoculated at the rate of two per cent growth media at 10,000 rpm for 15 min. Wells were bored on the indicator spreaded MHA Petri dishes using a sterile borer and were loaded with 50µl of both the CFS. The CFS loaded plates were incubated at 37 °C for 24 h and the zones of clearance (ZOC) developed around the wells were measured to assess the antimicrobial property of the isolates.

Assessment of impact of sucrose and dipotassium hydrogen phosphate supplementation on the antimicrobial activity of paneer whey CFS

Considering the lower antimicrobial activity exhibited by PW-CFS attempts were done to improve its potential as a growth medium by adding different levels of sucrose (Nice chemicals) as a carbon source and dipotassium hydrogen phosphate (Merck, Chemicals) as a buffering agent. The different levels of sucrose used were one, two, three, four and five per cent and dipotassium hydrogen phosphate levels were 0.1, 0.2, 0.3, 0.4 and 0.5 per cents. Production of antimicrobial agents by *Lb. fermentum* MH782089 grown in supplemented paneer whey (sPW) was assessed by checking the antimicrobial activity of the CFS of the sPW after 24h of incubation at 37 as mentioned earlier.

Optimisation of production of antimicrobial metabolites by *Lactobacillus fermentum* MH782089 in terms of temperature and period of incubation

In order to optimize the temperature and period of incubation overnight activated culture of the isolate was inoculated into sterile PW at the rate of two per cent and were incubated at two different temperatures (37 °C and 42 °C) for different incubation periods (24, 48, 72 and 96h). After the designated period of incubation, the CFS obtained by subjecting the contents to centrifugation at 10,000rpm for 15 min were

assayed for their antimicrobial potential against *S. aureus* by well diffusion assay.

Results and Discussion

Assessment of suitability of sterile paneer whey as growth medium for antimicrobial metabolite production

The growth media commonly used for the cultivation of Lactic acid bacteria is de Man Rogosa Sharpe (MRS) media. Considering the cost factor and the non- food grade status of this media use of sterile whey as a growth medium for the cultivation of *Lb. fermentum* MH782089 was attempted. Antimicrobial activity obtained upon growing *Lactobacillus fermentum* in sterilized paneer whey and MRS broth is given in Table 1. The smaller zone of clearance (ZOC) obtained for PW- CFS indicates that paneer whey is not effective as MRS broth in supporting the growth and metabolism of *Lactobacillus fermentum* MH782089. This is in agreement with the previous reports of lower activity of LAB in whey (Bulatovic *et al.* 2014) and contradictory to the earlier finding of sterilised paneer whey as an excellent media for the growth and metabolism of *Lactobacillus acidophilus* (Shafna *et al.* 2018) and could be attributed to the species difference. This is in agreement with the observation of Bulatovic *et al.* (2014) that species dependent variations occur in the growth and activity of LAB in whey. The observation of Shafna *et al.* (2018) that sterilised paneer whey is an excellent media for the growth and metabolism of *Lactobacillus acidophilus* is also in support of the species dependent variations in the suitability of paneer whey as a growth medium for LAB.

Table 1: Antimicrobial activity of *Lactobacillus fermentum* MH782089 in MRS broth and sterile paneer whey

Growth medium used	Zone of Clearance (mm, inclusive of well diameter 6 mm)
MRS-broth	9.6±0.5
Sterilized paneer whey	22.3±0.5

Assessment of impact of sucrose and dipotassium hydrogen phosphate supplementation on the antimicrobial activity of paneer whey CFS

Considering the essentiality of food grade materials for supplementation, this study attempted supplementation of paneer whey with sucrose and dipotassium hydrogen phosphate in order to make it compatible to the composition of MRS broth. On supplementation of paneer whey with different levels of sucrose and buffering agent an increase was observed in the antimicrobial activity against indicator organism *S. aureus* (Table 2, Fig. 1). It is clearly evident that supplementation of paneer whey with 3 percent sucrose and 0.3 percent dipotassium hydrogen phosphate resulted in the highest ZOC value. Lower as well as higher rates of supplementation resulted in decrease in antimicrobial activity. As LAB are fastidious in their nutrient requirements supplementation of their culture media with various growth factors are highly essential. Many other studies also have attempted the use of whey components for formulation of a cheaper growth medium for mass cultivation of LAB (Hanoune *et al.*, 2015, Aragón-rojas *et al.*, 2018) [5, 1]. Though supplementation of paneer whey with sucrose and dipotassium hydrogen phosphate resulted in some enhancement in the antimicrobial activity further studies are to be conducted to develop it as a compatible alternative for MRS broth.

Optimisation of production conditions for antimicrobial metabolites in terms of temperature and period of incubation

On assessing the antimicrobial activity of the CFS obtained by growing *Lactobacillus fermentum* MH782089 in supplemented paneer whey at different incubation temperatures and periods maximum effect was observed for sPW incubated at 37 °C for a period of 72h (Table 3). This observation is in agreement with that of Thalluri *et al.* (2017)^[9] for *Lactobacillus fermentum* MTCC No. 1745 and Mushood *et al.* (2012)^[7] for *Lactobacillus faecium* B3L3.



Fig 1: MHA plates showing zones of clearance around the wells loaded with sPW-CFS with different levels of the supplementation

Table 2: Effect of supplementation on antimicrobial activity of paneer whey-CFS

% Supplementation		Zone of Clearance (mm, inclusive of well diameter 6 mm)
Sucrose	Buffering agent	
1%	0.1%	9.6±0.5
2%	0.2%	14.6±0.5
3%	0.3%	16.6±0.5
4%	0.4%	12.3±0.5
5%	0.5%	9.6±0.5

Figures are mean±standard error of three replicates

Table 3: Zones of clearance obtained on incubation of *Lactobacillus fermentum* MH782089 inoculated sPW at different temperatures and periods

Temperature of Incubation	Period of Incubation	Zone of clearance (mm, inclusive of well diameter 6 mm)
42 °C	24h	15.3±0.5
42 °C	48h	18.3±0.5
42 °C	72h	20.3±0.5
42 °C	96h	18.3±0.5
37 °C	24h	17.6±0.5
37 °C	48h	19.6±0.5
37 °C	72h	22.6±0.5
37 °C	96h	13.6±0.5

Figures are mean±standard error of three replicates

Conclusion

Compared to the antimicrobial activity obtained in MRS broth, lower antagonistic activity was exhibited by *Lactobacillus fermentum* MH 782089 when sterilized paneer whey was used as the growth medium. But use of paneer whey supplemented with sucrose and dipotassium hydrogen phosphate was used as the growth medium an increase was observed in the antimicrobial effect of *Lactobacillus fermentum* MH 782089 metabolites. Optimization of production parameters like temperature and period of incubation also resulted in an increase in antimicrobial effect. From the observations it can be concluded that sterilized paneer whey supplemented with 3 percent sucrose and 0.3 percent dipotassium hydrogen phosphate could be further explored as a potential growth media for the production of

antimicrobial metabolites by *Lactobacillus fermentum* MH 782089. Highest antimicrobial effect was obtained upon incubation of *Lactobacillus fermentum* MH 782089 inoculated supplemented paneer whey at 37 °C for 24h. It can be concluded that *Lactobacillus fermentum* MH 782089 cultivated in sucrose and dipotassium hydrogen phosphate supplemented paneer whey produced metabolites with strong antimicrobial activity substantiating the potential of the supplemented paneer whey as a cheap growth medium.

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