MJS, Vol. 6, No. 1, January - June 2007, pp. 69-80
ISSN 0975-3311
https://doi.org/10.12725/mjs.10.6

STUDIES ON NISIN PRODUCTION BY ISOLATED LACTOBACILLUS SPECIES IN AQUEOUS TWO-PHASE SYSTEM

Namita Singh and S.K. Mandal**

ABSTRACT

Isolated Lactobacillus sp. *(from cheese whey) was examined for its ability to produce bacteriocin like nisin in submerged fermentation and aqueous two-phase system (ATPS) at pH 6.5, 30°C for 18 h fermentation using M17S medium with 5% (v/v) inoculum and the nisin yields were obtained 4500 IU/ml and 20216 IU/ml where as biomass yields obtained 19.55 mg/ml and 31.88 mg/ml respectively. The maximum yield of nisin (24800 IU/ml) and biomass (22.49 mg/ml) were obtained in ATPS (28% PEG_{6000} and 4%$MgSO_4 .7H_2O$ with 5-times concentrated M17 medium containing 0.5% lactose as carbon source) under similar conditions.*

* *Department of Biochemical Engg. and Food Technology, Harcourt Butler Technological Institute, Kanpur-208002, India. E mail- ranjeet_hbti@rediffmail.com*

Introduction

Presently, bacterial nisin is used in preservation of processed cheese spread and various canned foods (Broughton, 1990) to extend the self life of pasteurized milk and to control lactic acid bacteria in beer production (Taniguchi et al., 1994). It inhibits bacterial spore germination and growth of Gram (+) bacteria. This bioactive peptide, nisin which is active against Gram (+) bacteria and has a molecular weight of 3354 Da and composed of 34 amino acids (Kim, 1997). For this reason, it is widely used as a natural preservative (Vessoni Penna and Moraes, 2002).

Lactic acid bacteria exert lactic acid into the growth medium to the concentration which is inhibitory to their growth. Integration of product removal from the fermentation medium is a means to reduce the end product inhibition and thus increase the productivity (Dissing and Mattiasson, 1994). One such technique is the extractive fermentation using aqueous two-phase system (ATPS), which provides a biocompatible environment for the bacterial cells (Fan Ouyang and Bai, 2000).

Lactobacillus sp. isolated from cheese whey in our laboratory has been shown to produce significant amount of a bacteriocin like nisin. In this study, several parameters such as phase components, growth temperature, cultivation time, pH, carbon sources, nitrogen sources and PEG of different molecular weights are investigated for the production and optimization of this bacteriocin by isolated *Lactobacillus sp.*

Materials and Method

Bacterial strains

Lactobacillus sp. isolated from cheese whey was used in this study for nisin production. Screening of antibacterial activity of the isolates was done using *B. licheniformis* MTCC 1483 and estimation of produced nisin activity was done by *S. cremoris* NCIM 2179 as test organism.

Media and culture conditions

Isolated *Lactobacillus sp.* was grown on M17S medium containing (g/l); peptone,5; phytone,5; yeast extract,2.5; beef extract,5; sucrose,5; ascorbic acid, 0.5; disodium b-glycerophosphate,19 and 1 ml of 1.0 M $MgSO_4.7H_20$ at pH 6.5 and 30°C for 18-24 h.

ATPS medium was prepared by five times concentrated M17S (without sucrose and $MgSO_4.7H_2O$) along with 28% PEG_{6000} or PEG_{4000} and 4% $MgSO_4.7H_2O$. Concentrated sucrose solution was sterilized separately and added before inoculation so as to avoid browning.

Isolation and maintenance of *Lactobacillus* sp. was done on MRS medium containing (g/l); peptone, 10; beef extract, 10; yeast extract, 5; glucose, 20; K_2HPO_4, 2; sodium acetate, 5; triammonium citrate, 2; $MgSO_4.7H_2O$, 0.2; $MnSO_4.7H_2O$, 0.2; agar, 20, pH 6.2 -6.6, was sterilized at 121°Cfor 20 min and after cooling, incubated at 30-32°C for 24 to 48 h.

Maintenance of *S. cremoris* was also done on MRS medium where as medium for *B. licheniformis* contains (g/l); corn starch, 10; yeast extract, 2; peptone, 5; $MgSO_4.7H_2O$, 0.5; KH_2PO_4,0.5;NaCl,1.5;$CaCl_2$, 1.5; agar, 20; pH 7 and incubated at 37°C for 24 h.

Isolation of bacterial strains

Lactic acid bacteria (LAB) are known to occur in milk products, meat products, fruits and vegetables. Samples of curd, cheese whey, spoiled milk and cabbage leaves were collected from local market, brought to laboratory within 3 h of collection and immediately used for isolation purpose.1 ml of each sample was mixed in 10 ml sterile distilled water and several dilutions such as 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were done separately with 15 ml MRS agar medium for plating and incubated at 32°C for 48 h. Pink colored distinct colonies were isolated and grown on the fresh MRS agar medium to obtain pure culture.

Selection of bacterial strains on the basis of their antimicrobial activity

The antibacterial activity of the isolated culture of LAB was detected, following the 'agar spot' test using *B. licheniformis* as a test organism (Lewus and Montville, 1991). Among 10 different isolates, the antibacterial activity was determined on the basis of zone of inhibition. The isolated bacterial strain, producing maximum inhibition zone diameter (mm), was used for further studies (Sholera et al., 1998).

Inoculum preparation and cultivation

Isolated *Lactobacillus sp.* was inoculated into 20 ml of the cultivation medium (M17S) in a 100 ml Erlenmeyer flask and incubated at 30°C and 180 rpm for 24 h. A fresh culture of actively growing cells was always used as the inoculum. Fermentations were carried out in 250 ml Erlenmeyer flask containing 50 ml

production media along with 5%(v/v) inoculum. The flasks were incubated at 30°C and 180 rpm for 18 h without pH control. Samples were withdrawn at regular interval and analyzed for growth and nisin activity.

Growth determination

The fermentation broth was centrifuged at 8000 rpm for 10 min and the cells were washed twice with saline (0.9% NaCl) and dried for 90 min at 105°C. The cells grown were expressed in terms of dry weight basis (mg/ml).

Nisin activity determination

For estimating nisin activity, samples from both the phases were collected and pH was adjusted to 2.0 with a few drops of 5.0 M HCl. The samples were then heated in boiling water for 10 min, cooled to 30°C and appropriate dilutions (2 to 5 times) were made with 0.02M HCl (Kim et al.,1997). The nisin activity of the above diluted samples (clear supernatant) was determined by agar diffusion method (Bioassay) using *S. cremoris* NCIM 2179 as a test organism (Beach, 1952).

A standard curve (10 to 10,000 IU/ml, 900 IU=1 mg of pure nisin) was made using a stock solution of 10,000 IU/ml nisin ml^{-1}. The latter was prepared by dissolving 55.55 mg of nisin into 5 ml 0.02 N HCl. The activity of nisin from samples was determined and expressed in international units per ml (10^0 to 10^4 IU/ml). Each assay was performed in duplicate (Pongtharangkul and Demirci, 2004).

Results and Discussion

This paper reports on the potential use of aqueous two-phase system for cultivation of isolated *Lactobacillus sp.* to produce nisin.

Selection of the isolated strain

Only three strains I_1, I_2 and I_3 among ten isolated bacterial strain showed noticeable antibacterial activity (Table 1).

Of these three isolates, I_2 obtained from cheese whey gave maximum nisin activity of 3090 IU/ml and biomass of 16.50 mg/ml in M17S broth. However, I_3 from cabbage leaves showed optimum activity of 1080 IU/ml and biomass of 12.90 mg/ml. Isolate I_1 from spoiled milk gave minimum nisin activity of

560 IU/ml and biomass of 9.60 mg/ml. Thus the further experiments were carried out using isolate I_2 only.

Selection of salt to suit aqueous two-phase system formation and cell growth

Lactic acid bacteria can tolerate high osmotic pressure, using this property; we added various salts (5%) in M17 medium to test their effects on cell growth. Results are summarized in Table 2.

The data in the Table 2 indicate that out of four different salts tested, $MgSO_4.7H_2O$ allowed the maximum growth of isolated *Lactobacillus* sp. (12.87 mg/ml) whereas the control representing the M17S broth (without any added salt) produced the maximum growth of isolated *Lactobacillus sp.* (27.95 mg/ml) along with phase separation. Kpi^+ also resulted in better growth of bacterial cells as compared to other salts, but it was enable to create phase separation when added to ATPS media. Since $MgSO_4.7H_2O$ was the only salt in M17S medium for the maximum cell growth and hence it is concluded that $MgSO_4.7H_2O$ is the best salt to be used to form an ATPS with PEG.

Variation of salt concentration ($MgSO_4.7H_2O$)

The effects of $MgSO_4.7H_2O$ concentrations (3.5 to 8%w/v) on cell growth were examined employing M17S broth at initial pH 6.5, 30°C and 180 rpm for 18h.

The observed growth of cells is given in Table 3.

The results from Table. 3 indicate that growth of cells increased gradually with increasing salt concentration from 3.5 to 4% and maximum cell growth (20.60 mg/ml) attained with 4% salt concentration. On further, increasing the salt concentration (from 4-8 w/v %) the biomass yield decreased gradually and it reached to 5.21 mg/ml with 8% salt concentration. Thus result indicates that 4% $MgSO_4.7H_2O$ was suitable for maximum cell growth and nisin production by aqueous two-phase system. Phase separation was also visible with 4% salts concentration.

Effect of PEG molecular weight on cell growth and nisin production

The isolated *Lactobacillus* sp. grown with 28% PEG_{4000} or PEG_{6000} and 4% $MgSO_4.7H_2O$ in ATPS media at initial pH 6.5, 30°C and 180 rpm for 18 h and the results are given in Table 4.

Table 1. Comparison of nisin activity and cell growth obtained from isolated strains I_1, I_2 and I_3

Priomass Isolate	yield (mg/ml)	Nisin activity (IU/ml)
I_1	9.60	560
I_2	16.50	3090
I_3	12.90	1080

Table 2. Effect of various salts on bacterial cell growth, isolated *Lactobacillus sp.*

Salts	Concentration (w/w)	Biomass (mg/ml)
NaCl	5%	4.021
$MgSo_4.7H_2O$	5%	12.87
$MgCl_2.6H_2O$	5%	2.60
*Kpi +	5%	6.56
**Control	0	27.95

*$K_2H\ PO_4 + KH_2\ PO_4$ [1:1]

** M17S broth

Table 3. Effect of salt on phase separation and growth of isolated *Lactobacillus* sp.

Concentration of $MgSo_4.7H_2O$ (1% w/v)	Biomass (mg/ml)
3.5	12.20
4.0	20.60
4.5	18.10
6	9.66
8	5.21

From the data given in Table 4 it is found that biomass increases with increasing the molecular weight of PEG, since maximum biomass of isolated Lactobacillus sp. was obtained with PEG_{6000} (31.80 mg/ml) as compared to PEG_{4000} (26.65 mg/ml).

Nisin activity was also found to be affected by PEG molecular weight, since no phase separation was obtained with PEG_{4000} due to lower molecular weight (4000Da). Nisin activity was 20216 and 780 IU/ml with PEG_{6000} and PEG_{4000} respectively. It is found that nisin concentration was positively correlated with the molecular weight of PEG as the biomass yield and nisin production were increased with increasing molecular weight of PEG.

Nisin production by submerged fermentation and aqueous two-phase system (ATPS)

The fermentation was carried out separately in M17S broth and ATP media using freshly grown isolated *Lactobacillus* cells at initial pH 6.5, 30° C and 180 rpm for 18 h and the results obtained are indicated in Tables 5 and 6 respectively for submerged fermentation and ATPS.

Fermentation results from Table 5 and Table 6 indicate that maximum nisin yield of 20216 IU/ml was attained with isolated strain in ATPS, where as this yield was 4500 IU/ml in submerged fermentation. Similarly, bacterial cell growth was also higher in ATPS (31.67 mg/ml) as compared to submerged fermentation (19.55 mg/ml).

Effect of PEG_{6000} concentration on nisin production

The effects of different concentration of PEG_{6000} on cell growth and nisin production were examined employing ATPS media with 4% $MgSO_4.7H_2O$ and different concentration (11%-30%) of PEG_{6000} at initial pH 6.5, 30°C and 180 rpm for 18 h. The results are indicated in Table 7.

Results from Table 7 indicate that with increasing concentration of PEG_{6000} from 11 to 28%, biomass and nisin activity increased gradually but on further increasing PEG_{6000} concentration above 28 to 30%, there was a sharp decrease in biomass yield and nisin activity. The maximum nisin activity of (20216 IU/ml) was attained with 28% PEG_{6000} with clear phase separation where as moderate activities were obtained with 11%-18% PEG_{6000} but there was no phase separation observed. The amount of nisin produced was positively correlated with concentration of PEG_{6000} and the ratio of the top phase to bottom phase. The yield of nisin was

maximum when the volume of top phase was bigger that that of the bottom phase which was obtained with 28% PEG_{6000} and 4% $MgSO_4.7H_2O$.

Effect of pH on nisin production

Several experiments were performed to study the effect of pH values on nisin production by the strain under investigation in ATPS at 30°C and 180 rpm for 18 h. The pH values of the production media were taken in range of 4-8. The results are given in Table 8.

Table 4. Effect of polyethylene glycol (PEG) molecular weight on nisin production

PEG_{4000}			PEG_{6000}				
Final pH	Biomass yield (mg/ml)	Nisin activity a(IU/ml)	Final pH	Biomass yield (mg/ml)	Nisin activity (IU/ml)		
					Top phase	Bottom Phase	Average activity
5.81	10.64	780	5.60	31.80	14125	28183	20216.8

Table 5. Nisin production by isolated *Lactobacillus* sp. using submerged fermentation

Final pH	Biomass yield (mg/ml)	Nisin activity (IU/ml)
5.94	19.55	4500

Table 6. Nisin production by isolated *Lactobacillus* sp. using ATPS

Final pH	Biomass yield (mg/ml)	Nisin activity (IU/ml		
		Top phase	Bottom phase	Average
5.60	31.67	14125	28183	20216

Data from Table 8 indicate that with increasing pH value from 4 to 6.5, the nisin activities as well as biomass yields increased continuously However, the biomass yield (31.80 mg/ml) and nisin production (20216 IU/ml) by isolated *Lactobacillus sp.* were optimum at pH 6.5.On further increasing the pH value above 6.5 there was a sharp decline in both the nisin activity and biomass yield.

Effect of lactose and sucrose as carbon sources on nisin production in ATPS

Experiments have done to find the effect of lactose and sucrose on the biomass yield and nisin production in ATPS with 28%, PEG_{6000} and 4% $MgSO_4.7H_2O$ at initial pH 6.5, 30°C, and 180 rpm for 18 h fermentation. The results are given in Table 9.

From the Table 9 it is evident that sucrose promoted the growth of isolated *Lactobacillus* sp. (biomass yield 31.67 mg/ml) whereas lactose gave the maximum yield of nisin (24800 IU/ml). Control represents the ATPS medium without any carbon source.

Conclusions

Experimental data on the production of active bacteriocin like nisin have shown that its production is severely affected by various factors. The effects of phase component on phase separation in ATPS have also studied. These studies have shown that it is imperative to have an accurate quantification method to distinguish between growth promoting factors and those that actually affect nisin production. A comparison between submerged fermentation and ATPS indicates that ATPS is suitable for nisin production since the nisin yield is obtained 20216 IU/ml, which is approximately 4-folds to that of submerged fermentation (4500 IU/ml) with isolated *Lactobacillus* sp. Further, experimental results signify that $MgSO_4.7H_2O$ at a concentration of 4% (w/v) is suitable for cell growth (Table 3) and phase separation (Table 7).

On the variation of PEG_{6000} concentration it is found that maximum nisin activity is attained 20216 IU/ml with 28% PEG by isolated Lactobacillus sp. Phase separation is also visible with this composition hence, it is concluded that 28% PEG_{6000} and 4% $MgSO_4.7H_2O$ is suitable for phase separation and nisin production in ATPS (Table 7). It is also found that 0.5% lactose along with M17 medium in ATPS gave maximum yield of nisin 24800 IU/ml and similar conditions.

Table 7. Effect of PEG concentration on cell growth and nisin production

Conc. of PEG_{6000} (%)	Biomass yield (mg/ml)	Nisin activity (IU/ml)		
		Top phase	Bottom phase	Average activity
11	9.14	*	*	460
16	10.40	*	*	610
18	14.79	*	*	2180
24	18.80	3100	8800	2796
28	31.67	14125	28183	20216
30	20.90	2800	6650	3955

* No phase separation

Table 8. Effect of pH on cell growth and nisin production by isolated Lactobacillus sp.

Initial pH	Biomass yield (mg/ml)	Nisin activity (IU/ml)		
		Top phase	Bottom phase	Average activity
4	14.27	500	2000	980
5	21.92	4050	8600	5490
6	24.22	9500	24200	13243
6.5	31.80	14125	28183	20216
7	12.99	850	1150	950
8	6.66	210	540	314

Table 9. Effect of lactose and sucrose as carbon source on cell growth and nisin production

Carbon source (0.5%)	Final pH	Biomass yield (mg/ml)	Nisin activity (IU/ml)		
			Top phase	Bottom phase	Average activity
Control	6.20	6.80	50	520	191
Sucrose	5.60	31.67	14125	28183	20216
Lactose	5.40	22.49	14000	50000	24800

Studies on the variation of PEG molecular weight using PEG_{6000} and PEG_{4000} and the results indicate that there exist a positive correlation between the PEG molecular weight and nisin production. Phase separation is also visible with higher molecular weight of PEG (Table 4).

Thus the present studies on nisin production conclude that aqueous two-phase system provides a biocompatible environment for bacterial cells and a potential process for the extractive cultivation where the cells are confined to one phase and product in other phase. It is possible to cultivate the cells and extract lactic acid to reduce growth inhibition and enhance nisin accumulation using ATPS. This provides a technological solution to reduce end product inhibition. In view of attractive applications of nisin as preservative for food and feed items, these factors are to be considered for the design of bioprocess for efficient production of nisin.

References

1. Beach AS (1952) A agar diffusion method for the assay of nisin. *J Gen Microbiol* 6:60-63.

2. Broughton JB (1990) Nisin and its uses as a food preservative. *Food Technol* 44: 100-117.

3. Chandrapati S and O'Sullivan DJ (1998) Procedure for quantifiable assessment of nutritional parameters influencing nisin production by *Lactococcus lactis* subsp *lactis*. *J Biotech* 63: 229-233.

4. Dissing U and Mattiasson B (1994) Cultivation of *Lactococcus lactis* in a polyelectrolyte neutral polymer aqueous two-phase system. *Biotechnol Lett* 16 :333-338.

5. Fan Ouyang CL and Bai J (2000) Extractive cultivation of *Lactococcus lactis* using a polyethylene glycol/$MgSO_4$.$7H_2O$ aqueous twophase system to produce nisin. *Biotechnol Lett* 22:843-847.

6. Kim WS (1997) Nisin production by *Lactococcus lactis* using two-phase batch culture. *Lett in Appl Microbiol* 25:169-171.

7. Kim WS Hall RJ and Dunn NW (1997a) The effect of nisin concentration and nutrient depletion on nisin production of *Lactococcus lactis*. *Appl Microbiol Biotechnol* 48:449-453.

8. Lewus CB and Montville TJ (1991) Detection of bacteriocins produced by lactic acid bacteria. *J Microbiol Meth* 13:145-150.

9. Pongtharangkul T and Demirci A (2004) Evaluation of agar diffusion bioassay for nisin quantification. *Appl Microbiol Biotechnol* 65(3):268-272.

10. Sarkar PK and Banerjee S (1996) Antibacterial activity of lactic acid bacterial isolates obtained from natural habitates. *J Food Sci Technol* 33:231-233

11. Sholera Z Stefanova S and ChiperaV (1998) Screening of antimicrobial activities among *Bulgarian Lactobacillus Strain*. *J of Cult Collec* 2: 15-20.

12. Taniguchi M Hoshino K Urasaki H and Fujii M (1994) Continuous production of an antibiotic polypeptide (nisin) by *Lactococcus lactis* using a bioreactor coupled to a microfiltration module. *J Ferment Bioengg 77:704-708.*

13. Vesoni Penna TC and Moraes DA (2002) Optimization of nisin production by *Lactococcus lactis . Appl Bioch Biotech* 98-100: 775-789.