Enhancement of Nisin Production by *Lactococcus lactis* subsp. *lactis* MTCC 440 using a Novel Soya Permeate Medium

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One of the biggest problems when producing nisin from lactic acid bacteria on an industrial scale is the high cost of the complex peptide sources of the specific commercial media. Improving nisin production through optimization of fermentation parameters would make nisin more cost-effective for various applications. In this work the possibility of using soya permeate as a substitute for commercial peptide sources was studied. The growth and nisin production by Lactococcus lactis subsp. lactis MTCC 440 was investigated in fixed volume batch fermentation on soya permeate, and MRS medium. Kinetic parameters and nisin production was higher to those obtained with bactopeptone and commercial media. Soya permeate was also supplemented with growth stimulating amino acids. Nisin biosynthesis is strongly dependent on the presence of a sulphur source, either an inorganic salt (magnesium sulphate or sodium thiosulphate) or the amino acids methionine, or cysteine. The amino acids serine, threonine and cysteine highly stimulate nisin production without affecting the final cell yield, indicating their precursor role during nisin biosynthesis.

Key words: *Lactococcus lactis* subsp. *lactis, B*atch fermentation, Nisin, Soya permeate.

1. INTRODUCTION

Nisin, antimicrobial peptide (3.4 kDa), is produced by *Lactococcus lactis* subsp. *lactis* during its exponential growth phase [1]. Nisin is a bacteriocin commercially used as natural agent for food biopreservation. It has recently been considered safe by the World Health Organization (WHO) and by the Food and Drug Administration (FDA), with the denomination of generally recognized as safe (GRAS) [2,3].

Nisin has large antimicrobial activity spectrum against Gram-positive bacteria and their spores but shows little or no activity against Gram-negative bacteria, yeasts or moulds. However, activity of nisin can be enhanced by combination with surfactants chelating agents and adjuvant [4, 5]. As a result of its antimicrobial properties, it has been accepted as a safe and natural preservative in different areas of food industry and is being considered for use in both the pharmaceutical and veterinary areas and as a therapeutic agent [6,7,8].

One problem associated with the growth of lactic acid bacteria and production of bacteriocins on an industrial level, especially for applications based on their probiotic effects, is their high cost that is due to their demand for diversified peptide sources, which several commercial media {Man Rogosa and Sharpe (MRS), all purpose with Tween (APT), Elliker broth, tryptone glucose extract (TGE), trypticase soy broth (TSB) and brain heart infusion (BHI) *etc*}, resolve by including products such as bactopeptone, triptone, meat extract or yeast extract (sometimes all of these) in formulations [9] and using by-products from the food industries as a basis of the culture media, such as dairy whey [3,6,10,11,12] mussel-processing wastes [13] and waste protein sources from octopus [9]. Two ways to reduce this cost are the utilization of cheaper fermentation substrates and the determination of the optimum parameters such as media composition, temperature and pH *etc* for high nisin production.

Peptones as water soluble protein hydrolysates which are not coagulated by heat. Peptone from soya are uncommon, despite their good yield in areas such as the growth of nisin producing *Lactococci* in cooked rice supplemented with soyabean extract [14], evaluation of lactic starter cultures in soymilk [15], effect of soyabean products on the glucose and Cl^- transport capacity in porcine small intestine [16], effect of fermented soyabeans on diarrhoea and feed efficiency in weaned piglets [17].

In this paper, we investigated the use of soya permeate as a protein source for the production of nisin. Two media we used as terms of comparison: commercial MRS (usual for the cultured of lactic acid bacteria) and another in which the soya permeate was replaced by commercial bactopeptone. The comparison of the results demonstrated the efficiency of the soya permeate for the nisin production.

2. MATERIALS AND METHODS

Bacterial strains: *Lactococcus lactis* subsp. *lactis* MTCC 440, the nisin producing strain and *Lactococcus lactis* subsp. *lactis* MTCC 3038, the target organism, were obtained from the Microbial Type Culture Collection MTCC (IMTECH, Chandigarh, India). Strains were grown in MRS (de Man, Rogosa and Sharpe medium) broth and maintained as frozen stocks held at -20°C in MRS broth containing 30% (v/v) glycerol. Working cultures were maintained as slant on MRS agar at 4°C and subcultured twice in liquid cultures in the same medium at 37°C before use.

Fermentation medium: In the present study a different type of fermentation media Supplemented soya permeate (SSP) was tested for nisin production from *Lactococcus lactis* subsp. *lactis* MTCC 440. The composition of the media used is shown in Table 1. For comparison, a medium was used (Medium M), where soya permeate was replaced by a commercial bactopeptone solution, with an equivalent level of protein, as well as a commercial MRS medium. In all cases, the initial pH was adjusted to 7.0.

SSP media was also supplemented with growth stimulating amino acids (alanine, arginine, cysteine, glutamic acid, histidine, leucine, methionine, serine, threonine, valine) and was tested for growth of *Lactococcus lactis* subsp. *lactis* MTCC 440 and nisin production.

Table 1: Composition of media used in studies (g/l)

Media Composition	MRS	SSP	M
Glucose	20.0	20.0	20.0
Yeast extract	5.0	2.0	2.0
Sodium acetate	5.0	3.0	3.0
Ammonium citrate	2.0	2.0	2.0
KH_2PO_4	2.0	2.0	2.0
$MgSo_4$	0.2	0.2	0.2
$MnSo_4$	0.05	0.05	0.05
Tween 80	1 ml	-	-
Beaf extract	10.0	-	10.0
Peptone	10.0	-	10.0
Defatted Soya flour	-	10.0	-

Inoculums preparation and fermentation conditions: A pre-inoculum was prepared by transferring a loopful of cells from a 24 h old MRS slant into 10 ml of MRS broth and incubated for 12 h at 37°C. An aliquot (1ml) of this preculture was used to inoculate 40 ml of MRS medium in a 250 ml Erlenmeyer flask, which was cultivated with shaking at 150 rpm for 12 h at 37°C and then used as the inoculum. All batch cultures were inoculated with a 2% (v/v) of this inoculum.

Batch fermentations on SSP and MRS were carried out at a controlled temperature of 35°C in a 2 litre bench top fermenter (LABFORS ® INFORS AG, Switzerland) with an agitation of 150 rpm at pH 7.0. The fermenter was filled with 1 l working volume of medium. The aeration level (0.5 l/h) was obtained by controlling the air supply by a flow meter. The samples (100ml) were withdrawn at regular intervals (each 4 h) to determine the cell growth, antibacterial activity and analytical determinations.

Analytical methods: Growth (biomass) was monitored by measurement of absorbance at 620 nm in a spectrophotometer (CECIL, ALPHA Series, England) and converted into cell dry weight. Cells were harvested by centrifugation (5,000 rpm for 15 min. at 4°C) of culture samples and washed twice with saline (0.9% NaCl). The culture supernatants were used for analytical determinations. Total sugars (TS) were determined by using the DNS method and protein was measured by method of Lowry *et al*.,1951 [18].

Nisin activity determination: Antimicrobial activity secreted into liquid medium was detected by agar- well diffusion method [19]. MRS soft agar (5ml) inoculated with 1% (v/v) of an indicator sensitive strain overnight culture was overlaid on an agar plate. After cooling, wells (6 mm diameter) were punched in the agar plates and filled with 50 µl of CFF. After incubation overnight, the antimicrobial activity was expressed as the diameter of the zone of inhibition around the wells. Bacteriocin activity was assayed by two fold dilution of crude bacteriocin in terms of arbitrary unit (AU). Arbitrary unit was defined as the reciprocal of the highest dilution which showed a clear zone of inhibition [20].

3. RESULTS AND DISCUSSION

The pattern of nisin production by *Lactococcus lactis* subsp. *lactis* 440 in SSP media is presented in Fig. 1. and it was compared to MRS and M media The culture grew with almost constant rate upto 36 h but maximum growth was obtained after 24 h of incubation period it was 0.22 in terms of O.D. at 620 nm while it was less (0.20 and 0.18) in case of MRS and M media respectively (Fig. 1 A). The growth was accompanied by production of acid as evidenced by fall of pH from 7.0 to 5.0 after 24 h of incubation period (Fig.1B). In an attempt to clarify the role of pH in nisin production, three series of cultures were performed for *L. lactis* subsp. *lactis* 440 on SSP medium. SSP was buffered at pH 7.0 with 0.03, 0.10 and 0.25 M NaOH. In *L. lactis* subsp. *lactis* 440 the increase in buffer concentrations determined, as excepted, different acidification rates (data has not been shown). Although no significant differences in cell growth were found amongst the cultures buffered at different concentrations but nisin production was affected. In *L. lactis* subsp. *lactis* 440, the highest nisin production was obtained in the SSP medium buffered with 0.1 M NaOH (final pH 5.0). This specific effect of pH on nisin production could be reached to the final pH value reached in the cultures. Similar observations on the existence of an optimum final pH value in bacteriocin have been described previously for nisin production by *L. lactis* subsp. *lactis* CECT 539 [13,21]. It has been reported that the rate of acidification also had an effect on the production of nisin [13, 22]. From these observations, it can be pointed out that the increase in the acidification rate of the SSP medium enhances the nisin production, before a final pH value unsuitable for cell growth of *L. lactis* subsp. *lactis* MTCC 440.

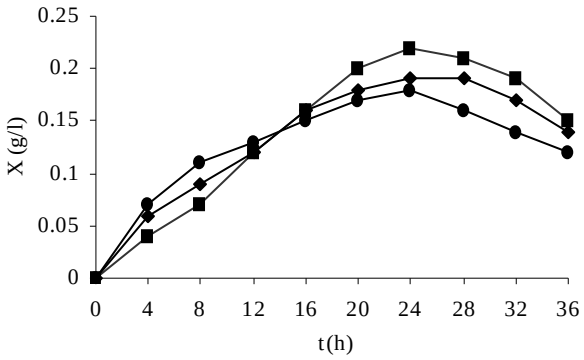

Fig. 1 A: Growth of *Lactococus lactis* subsp. *lactis* MTCC 440 on supplemented soya permeate (SSP: ■ MRS: ● and M: ♦ media)

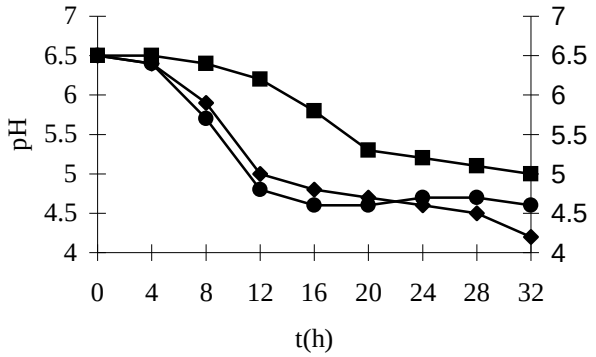

Fig. 1B: Influence of pH on cell growth and nisin production from *Lactococcus lactis* subsp. *lactis* 440 on different media (SSP: ■ MRS: ● and M: ♦)

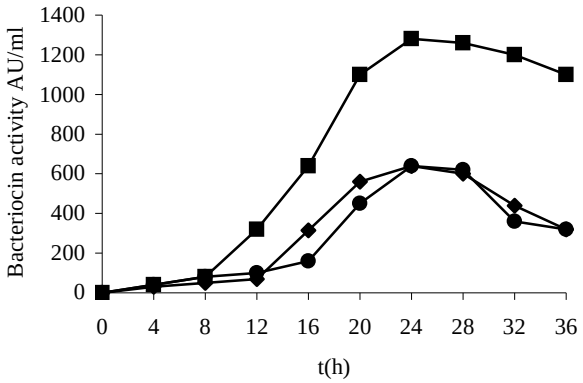

Fig. 1 C: Nisin activity (AU/ml) on different media (SSP: ■ MRS: ● and M: ♦)

The pattern of antibacterial activity indicates that production of nisin was started after 2 h and increased rapidly till maximum activity of 1280 AU/ml was reached after 24 h of incubation period (Fig.1C). Thus the rate of biomass of *L. lactis* subsp. *lactis* MTCC 440 and the nisin production by this strain, both were higher than those obtained with the commercial media. The peptone source from SSP medium gave better results that the commercial media in the production of nisin and biomass by *L. lactis*. In another words we can say that this source of peptone is a substitute for commercial peptones, for MRS medium in nisin production.

To formulate soya permeate medium and to evaluate the nutritional requirements of the individual medium constituents, growth was measured in the SSP medium described above, changing the concentrations of its components and by adding some amino acids and vitamins. Addition of 2% yeast extract in SSP medium showed the maximum

bacteriocin production to 1280 AU/ml. There was no change in bacteriocin activity by addition of 0.5% and 1% yeast extract. Increasing the concentration of glucose, $MgSo_4$, KH_2PO_4 and Sodium acetate in SSP medium had no effect on bacteriocin activity. Addition of 1% Tween 80 increased the bacteriocin production and maximum activity of 2560 AU/ml was attained in this medium. However, there was no effect of addition of 1-3% sucrose, 0.5-2.0% lactose and 0.25-1.5% tryptone in the SSP medium as shown in Table 2.

Table 2: Nisin activity by *Lactococcus lactis* subsp. *lactis* MTCC 440 with different substitutes in SSP medium.

SSP substitutes	Concentration	Biomass (O.D.at 700 nm)	Nisin Activity (AU/ml)
SSP medium		1.0	1280
SSP + Yeast extract	0.05%	0.8	640
	1.0%	0.8	640
	2.0%	1.0	1280
SSP + Glucose	2.0%	1.0	1280
	2.5%	0.9	1280
	3.0%	0.9	1280
SSP + $MgSO_4$	0.025%	1.0	1280
	0.05%	0.8	1280
SSP + Tween 80	0.1%	1.1	1280
	1.0%	0.8	2560
	2.0%	0.8	2560
SSP + KH_2OPO_4	0.2%	1.1	1280
	0.4%	1.0	1280
SSP + Sod. Acetate	0.2%	1.0	1280
	0.4%	0.9	1280

During this study, SSP medium was also formulated, consisting growth stimulating amino acids (alanine, arginine, cysteine, glutamic acid, histidine, leucine, methionine, serine, threonine and valine) as shown in Table 3.

Table 3: Influence of amino acids on cell growth and nisin activity of *Lactococcus lactis* subsp. *lactis* MTCC 440 in SSP medium.

Media Composition	Biomass in terms of O.D. at 620 nm	Nisin activity AU/ml
SSP	0.22	1280
SSP + 0.1% Alanine	0.24	1280
SSP + 0.5% Alanine	0.18	640
SSP + 1.0% Alanine	0.12	320
SSP + 0.1% Arginine	0.23	5120
SSP + 0.5% Arginine	0.18	1280
SSP + 1.0% Arginine	0.10	640
SSP + 0.1% Cysteine	0.21	5120
SSP + 0.5% Cysteine	0.20	2560
SSP + 1.0% Cysteine	0.18	640
SSP + 0.1% Glutamic acid	0.24	5120
SSP + 0.5% Glutamic acid	0.20	5120
SSP + 1.0% Glutamic acid	0.16	1280
SSP + 0.1% Histidine	0.20	1280
SSP + 0.5% Histidine	0.21	640
SSP + 1.0% Histidine	0.17	640
SSP + 0.1% Leucine	0.23	1280
SSP + 0.5% Leucine	0.22	640
SSP + 1.0% Leucine	0.17	320
SSP + 0.1% Methionine	0.23	5120
SSP + 0.5% Methionine	0.22	5120
SSP + 1.0% Methionine	0.20	1280
SSP + 0.1% Serine	0.24	5120
SSP + 0.5% Serine	0.24	2560
SSP + 1.0% Serine	0.18	1260
SSP + 0.1% Threonine	0.24	2560
SSP + 0.5% Threonine	0.18	1260
SSP + 0.1% Threonine	0.20	640
SSP + 0.1% Valine	0.23	2560
SSP + 0.5% Valine	0.20	1280
SSP + 1.0% Valine	0.16	640

From these experiments, it could be concluded that the amino acids were essential for growth of *L. lactis* susp. *lactis* MTCC 440 at normal or maximal levels. Indeed, the omission of one of them still allowed growth at a very low rate, clearly indicating that *L.*

lactis susp. *lactis* MTCC 440 possesses the genes for the biosynthesis of these amino acids. The amino acids alanine, histidine and leucine did not showed significant effect on nisin production while others arginine, cysteine, glutamic acid, methionine, serine, threonine and valine gave nisin activities comparable with those obtained in SSP medium without adding the amino acids. The amino acids serine, threonine, and cysteine highly stimulated nisin production , clearly indicating their precursor role during nisin biosynthesis. Omission of glutamic acid allowed very less growth and nisin production underlining its absolute requirement for growth and nisin production. No medium was found that supported growth without nisin production or vice versa. The amino acid auxogram of *L. lactis* susp. *lactis* MTCC 440 was comparable with that of others *Lactococcus* strains (23, 24).

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