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# Nisin Production in a Two Liters Bioreactor Using *Lactococcus lactis* NCIM 2114

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## ABSTRACT

**The facultative anaerobe, *Lactococcus lactis* was grown in batch fermentation (1.5 L working volume) at 30°C, using 5% inoculums to initiate growth for the production of the antimicrobial food additive nisin. *L. lactis* strain NCIM 2114 produced a maximal nisin concentration of 50,400 IU/mL and 8,830 IU/mg biomass in MRS growth medium using a 2 L bioreactor with optimal conditions of 25 rpm and 0.2 vvm determined by a Box Behnken design statistical package. Nisin was obtained from cell-free extracts and quantified using ultra-fast liquid chromatography (UFLC). Maximum bioactive nisin concentration was found at 4 h and harvested. Increasing aeration, agitation or time until harvest had negative impact on bioactive product yield. In a repeated trial under the same conditions, cell-free extracts of nisin were measured by well diffusion assay at 240 IU/mL (antimicrobial activity). Bioactive nisin was confirmed. The optimal specific growth rate for *L. lactis* was measured to be 0.25 h$^{-1}$. This study shows that active biomass production can be increased using conditions determined by standard statistical methods.**

**Key words:** Nisin, Food preservative, RP-UFLC, Fermentation technology, Bioreactor, Bioactivity, Cell-free extract, Box Behnken.

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## INTRODUCTION

Nisin is a growth-associated metabolite (Liu and Hansen, 1990) produced by *Lactococcus lactis* and is used in the food industry and many biotechnological applications (Kong and Lu, 2014). Antimicrobial preservatives including nisin are part of the more than $ 22 billion global food additives market (Eluned et al., 2005). During 2013 to 2014, India alone, imported nisin to a value of ~ Rs. 2 million. However, current technologies provide only slow nisin production by *L. lactis* (Kaletta and Entian, 1989). To increase nisin production there is need to develop optimized conditions before the process is scaled up. Nisin production has been reported in batch (Vuyst and Vandamme, 1992; Wolf-Hall et al., 2009), fed batch (Papagianni and Avramidis, 2012) and continuous (Ömer, 2014) modes of fermentation. In batch fermentations with *L. lactis* ATCC 11454, under pH control, a maximum nisin titer of 3,100 IU/ml was obtained after 8 h of growth under moderate aeration using 75 g/L initial glucose in chemically defined medium (Papagianni and Avramidis, 2012). Fed-batch studies

showed that nisin production increased as a secondary metabolite if a stepwise-pH profile was imposed on the culture (Cabo et al., 2001).

In stationary batch fermentation mode without pH control in MRS medium, a nisin titer of 9,100 IU /mL in crude cell-free extracts was obtained (Singh et al., 2013) with a final pH 4.4 after 17 h growth. Such nisin producing cultures with value-added ability to produce acidity (lactic acid) in the medium can be used in traditional Indian lactic-fermented vegetable drinks. In these cases the fermentation process with native lactic acid bacteria (Sethi, 1990) is completed in 6 to 7 days. Therefore, optimized growth conditions for *L. lactis* in batch modes will add value to lactic drinks containing nisin as an antimicrobial (Karovicova et al., 1999). Whereas the antimicrobial efficacy of nisin varies with indicator strains (Yoneyama et al., 2008), the specific methods used for measuring nisin content may not always be proportionate (Ripoche et al., 2006). There are few reports on aeration and agitation design for optimization of nisin production. Considering pH gradient effects, the time to harvest nisin can be optimized in batch mode. Aeration studies should also be coupled with agitation rate for nisin production. This study aims to optimize three factors (aeration, agitation and time of harvest), for producing optimal bioactive product and culture biomass from *L. lactis* in a batch process.

## MATERIALS AND METHODS

### Bacterial Strains and Culture Conditions

The bacterial strain *Streptococcus lactis* NCIM 2114, a nisin producer now classified as *L. lactis* (Schleifer et al., 1985) a homo-fermentative bacterium, was procured from National Collection of Industrial Microorganisms, NCL, Pune, India, and maintained by bimonthly sub-culturing. A 48 h growth of culture in de-Man Rogosa Sharpe (MRS) agar (De Man et al., 1960) slants (incubated at 30°C), were preserved at 4°C. The MRS medium used for growth and nisin production contained (g/L): proteose peptone 10; beef extract 10; yeast extract 5; dextrose 20; polysorbate 80 1; ammonium citrate 2; sodium acetate 5; magnesium sulphate 0.10; manganese sulphate 0.05; di-potassium phosphate 2.0; and agar 15.0 (when required for slants/ plates), with a final pH 6.5± 0.2. The medium was autoclaved at 121°C for 20 min. *Micrococcus luteus* B 287 was procured from NRRL Peoria, USA and used as a nisin-sensitive indicator species in assays of nisin bioactivity. Stock cultures were maintained at -4°C in nutrient agar and sub-cultured bimonthly. A uniformly dispersed starter inoculum of *L. lactis* NCIM 2114 in MRS broth having an OD of ~ 6 (at 600 nm), was inoculated at 5% for a step-wise scale up, from 75 to 1500 mL medium. The working medium volume was always 50% of a total vessel capacity.

### Batch Fermentation and Growth

For growth and nisin production from *L. lactis* NCIM 2114 strain, batch fermentation runs were monitored for 24 h, in a computer-controlled autoclavable bioreactor system (STR Sartorius® A+, Germany) containing 1.5 L medium (with inoculum). The fermenter maintained the temperature (30°C) and impellor agitation (rpm). In all experiments pH was allowed to change during fermentations. Aeration air-flow rate (vvm) was adjusted according to experiments. A dissolved oxygen probe (Hamilton, USA) monitored $pO_2$ % (Rowe et al., 2003). Sterilized antifoam was added as needed. Bacterial growth data was monitored using an Optek® NIR sensor which can record data at 800 nm. Concentration Units (CU) data were collected by an Optek® data logger (Model FC 20, USA). These data points represented the growth curve at 15 min intervals.

An indirect method (Mukhopadhyay, 2007) was followed to determine cell dry biomass. Three harvest periods (4, 7 and 24 h) of each batch run were also monitored for biomass. Biomass harvested at 24 h after initiation of each batch run, was washed twice with saline (0.85%) and centrifuged to a pellet and weighed. A Spectrophotometer (Beckman model DU 640, USA) was used to measure $OD_{600}$. This study was designed for 12 experiments using a Box Behnken design with the combination(s) that comprised of 3 parameters (harvesting time, aeration and agitation) (Behnken et al., 1972). The 12 experiments were conducted according to: (a) harvest periods: 4, 7 and 24 h; (b) aeration: 0, 0.2 and 0.4 vvm and (c) agitation: 25, 50 and 100 rpm represented in Table 1 in the Results section.

### Bioactivity of Nisin

Bioactivity (antimicrobial property) of nisin was measured using *Micrococcus luteus* as the sensitive indicator organism by agar well diffusion assay (Wolf and Gibbons, 1996). The standard nisin used for analysis was initially dissolved in 0.02 N HCl. Milk proteins that may be present in the standard were precipitated by centrifugation. Using the properties of nisin solubility and stability at low pH (Davies et al., 1998; Kelly et al., 2000), the nisin cell-free extracts obtained as described are suitable to be quantified. A calibration curve was prepared by a series of known standard nisin concentrations between 18 to 93 IU (from a 930 IU /mL stock standard solution). Total nisin produced in the bioreactor was quantified by UFLC and its bioactive component was determined using a standard calibration line of bioactive nisin vs inhibition zone areas. Total protein was determined using a bicinchoninic acid protein assay method (Wiechelman, 1988) at 562 nm after

**Table 1.** Nisin and biomass produced under different fermentation parameters using 2 L Sartorius stedim bioreactor.

| Parameters | | | Nisin | | Biomass |
|---|---|---|---|---|---|
| Harvest time (h) | Aeration (vvm) | Agitation (rpm) | IU/mL | IU/mg Biomass | g/L |
| 4 | 0 | 50 | 21465.18 | 3242.474 | 6.62 |
| 4 | 0.2 | 25 | 50390.67 (maximum) | 8830.141 (maximum) | 5.71 |
| 4 | 0.2 | 100 | 9987.78 (minimum) | 2259.679 | 4.42 |
| 4 | 0.4 | 50 | 17411.46 | 2638.1 | 6.6 |
| 7 | 0 | 100 | 24407.79 | 3194.737 | 7.64 |
| 7 | 0 | 25 | 17557.98 | 1925.217 (minimum) | 9.12 |
| 7 | 0.4 | 25 | 30256.38 | 1938.269 | 15.61 |
| 7 | 0.4 | 100 | 30183.12 | 3555.138 | 8.49 |
| 24 | 0 | 50 | 21196.56 | 3306.796 | 6.41 |
| 24 | 0.2 | 25 | 40305.21 | 3079.084 | 13.09 |
| 24 | 0.2 | 100 | 25225.86 | 1975.4 | 12.77 |
| 24 | 0.4 | 50 | 33101.31 | 2306.711 | 14.35 |

incubation of reaction mix for 30 min at 60°C. This method verified total nisin content (Ripoche et al., 2006).

### Oxygen Uptake Rate (OUR) and Total Reducing Sugars (TRS) Utilization

The OUR (µmoles $O_2$/L-h) of *L. lactis*, was determined (Rowe et al., 2003) at 25 rpm and 0.2 vvm between 0 to 8 h. The cell-free media was also analyzed for total reducing sugars (TRS) at hourly harvests (Ghose, 1987). Glucose (as TRS) utilization by *L. lactis*, was calculated against the initial levels in the medium.

### Antimicrobial Activity Assay for Bacteriocin Units ($ID_{50}$)

To quantify the inhibitory concentration of nisin produced by *L. lactis* NCIM 2114, a suspension of the indicator organism *M. luteus* B 237 with less than 12 h old growth in nutrient broth (NB) medium was obtained. The culture suspension was centrifuged and pellet re-suspended in NB buffered at pH 6·0 (Cabo et al., 1999). A bacteriocin unit for *L. lactis* was quantified by inhibition assay against *M. luteus* growth at $OD_{600}$. Cell-free extract of *L. lactis* was obtained after 48 h growth. The suspension of the indicator was mixed in an equal ratio (1:1) with different serial dilutions of nisin cell-free extract. The serial dilutions of nisin were: 1:2; 1:4, 1:8; 1:16; 1:32; 1:64, prepared in the biphthalate–NaOH buffer (0·05 M, pH 6·0). The mixtures were incubated for 5 h against control. As a control, the sample nisin was replaced by the same buffer medium. To quantify inhibitions changes in $OD_{600}$ of *M. luteus* were observed at 0 h and after 5 h of incubation. Taking $A_s$ to be the $OD_{600}$ difference for the sample and $A_c$ for that of the control (without nisin cell-free extract), the proportion of inhibition was calculated according to the formula

$$Inhibition = 1 - \frac{A_s}{A_c} \qquad (1)$$

The concentration of each nisin sample vs the proportion of inhibition 'I' was plotted to determine $I_{50}$ value.

## RESULTS

### Production of Nisin

The maximum nisin production was observed at 25 rpm, 0.2 vvm and 4 h harvesting time (Table 1). Nisin is soluble at acidic pH but it is inactivated at high pH as a result of denaturation, chemical modification (oxidation) or a combination of both (Liu and Hansen, 1990). The data indicates that aeration may affect nisin content by oxidation. Nisin concentrations in cell-free extracts measured by HPLC, ranged from ~ 9,988 IU/mL to 50,391 IU/mL. Nisin biomass ranged between 1,925 to 8,830IU/mg (Table 1).

### Bioactivity of Nisin by Well Diffusion Assay

Bioactive pure nisin produced by *L. lactis* assayed at 74 $mm^2$/IU between 150 to 750 µg/ 100 µL extract against *M. luteus* as indicator organism with an $R^2$ value 0.98 on the line fit. Whereas, when nisin was assayed between 1 to 15 µg/100 µL in wells, the $R^2$ value reduced to 0.85 and sensitivity was 98 $mm^2$/IU. The bioactivity of nisin produced in the bioreactor was measured with a

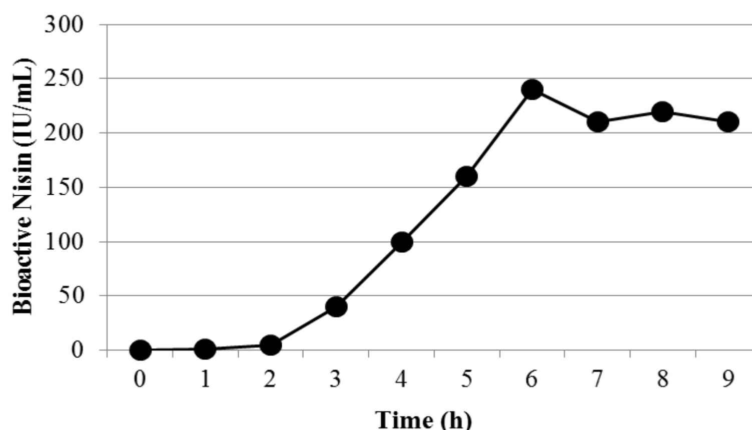


**Figure 1.** Bioactive nisin concentration during the growth of *L. lactis* (0.2 vvm x 25 rpm).

calibration line fit curve having an $R^2$ value of 0.98. This method gave same results when used within a range of 10 to 25 mm radius of inhibition zone towards *M. luteus* B237 (data not shown). This range of zone size was also found repeatable in a previous study using *L. lactis* NCIM 2114 against *Streptococcus agalactiae* as indicator strain (Singh et al., 2013). Nevertheless, content of nisin determined by UFLC was high. It is suspected that antimicrobial property of nisin protein was diminished by oxidative conditions in the bioreactor. It has also been reported that a diffusion assay may not always be proportionate to HPLC quantification due to lower sensitivity caused by long assay time (Ripoche et al., 2006).

### Antimicrobial ActivityAssay for Bacteriocin Units ($ID_{50}$)

Nisin bioactivity varies with indicator strains (Yoneyama et al., 2008). Nisin bacteriocin units ($ID_{50}$) were quantified using optimized procedures according to Cabo et al. (1999). A dose response curve showed that a 16 fold diluted nisin extract (data not shown) inhibited the indicator strain by 50 % (I = 0.5, Equation 1). One bacteriocin unit of *L. lactis* required 5 h exposure time to inhibit *M. luteus* B237 in the assay. Nisin cell-free extract product should be protected from oxidative damage. This is important to obtain active, high purity for practical applications. The work demonstrated that well diffusion assays are reliable for measuring the nisin bioactivity (Pongtharangkul and Demirci, 2004). *M. luteus* B237 has not been universally used as indicator organism (Rogers and Montville, 1991). The study suggests that batch fermentation may prevent high oxidation. Nisin oxidation cannot be ruled out as an important production concern. The shoulder peaks observed in UFLC may be an indication of product oxidation. This study demonstrated that aeration, agitation and harvesting time can affect nisin production (Singh et al., 2013). In this study the highest bioactive nisin concentration reached 240 IU/mL (Figure 1) in agar diffusion assay at 5 h during exponential growth phase with 0.2 vvm and 25 rpm.

### Oxygen Uptake Rate (OUR) and Total Reducing Sugars (TRS) Utilization

The oxygen uptake rates of the organism increased during growth of *L. lactis* (Figure 2). The bioactive nisin yielded by the organism was 31 IU/mg of glucose utilized at 5 h of growth when ~37% glucose was utilized from the medium. This yield decreased to 17 IU/mg after 8 h of growth even though 57% glucose was utilized from the medium. Carbon conversion rates increased as oxygen uptake rates increased (Papagianni and Avramidis, 2012). Since nisin is sensitive to oxidative damage (Wilson-Stanford et al., 2009; Kuipers et al., 1992), the yield of bioactive nisin showed sensitivity to oxygen. The data supports the conclusion that aeration can decrease nisin yields since the factors 0.2 vvm and 25 rpm increase oxygen uptake rates during the 0 to 8 h of growth period (Figure 2) with low bioactive nisin (240 IU/mL).

## DISCUSSION

Oxygen can affect both the health and mortality of aerobic microorganisms. The bacterium *L. lactis* is a facultative anaerobe, and under most conditions oxygen has negative effects on both its growth and survival. The effects of oxygen on lactic acid have been examined with *L. lactis*, a gram-positive facultative anaerobe (Duwat et al., 2001). This study demonstrates that active nisin biomass production from the fermentation of *L. lactis*

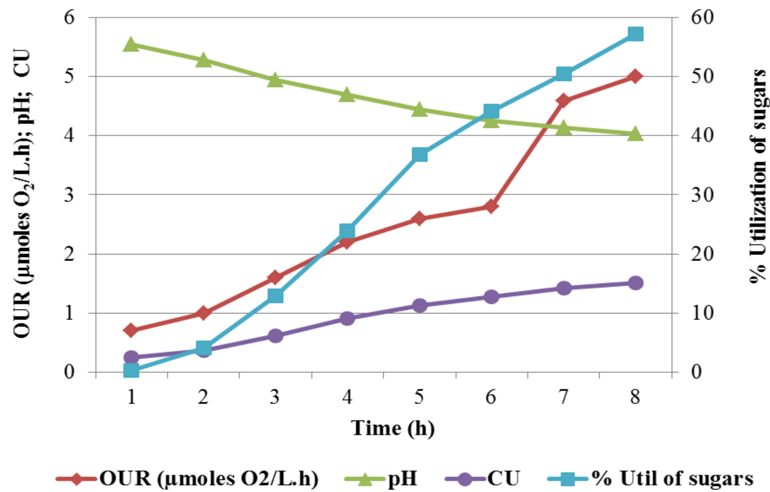


**Figure 2.** Oxygen uptake rate (OUR) and sugar utilization pattern during the growth of *L. lactis* (0.2 vvm x 25 rpm).

NCIM 2114 can be significantly increased by maintaining low dissolved oxygen levels. Table 1 shows that production of active nisin is inhibited by oxygen, due to oxidation of product. In this study, active nisin yield was maximized (>8,800 IU/mg biomass) at 4 h, 0.2 vvm and impellor agitation at 25 rpm. Interestingly, longer fermentation runs (up to 24 h) also had negative effect on active product yield. Similarly, increasing agitation (effectively increasing dissolved oxygen) had the effect of lowering nisin product yield. Finally, the negative effect of increased dissolved oxygen is further supported by the data when aeration rate (vvm) was increased to 0.4 vvm. The bacteria produce very low nisin values when aeration was reduced to nil levels. Fermentation-produced nisin was assayed in two standard ways: a. well diffusion assay and b. spectrophotometric antimicrobial assay against *M. luteus*. Both assay protocols indicated that bioactive nisin was sensitive to aeration as determined by HPLC. Aside from product oxidation, *L. lactis* responds as predicted when increasing dissolved oxygen. Figure 2 demonstrates that the bacteria increased (CU) only slightly and percent of sugar utilization increased with oxygen uptake rate (OUR). A potential application of *L. lactis* NCIM 2114 is its use as a starter culture for nisin production. This study demonstrated that biomass can be significantly increased by factor optimization. It was found that aeration control will optimize nisin yield and reduce nisin oxidation. However, such responses may be strain specific (Kim et al., 1997). In future studies, continuous mode of fermentation will be investigated. Nisin can be harvested intermittently during fermentation (Pongtharangkul and Demirci, 2007). Continuous mode production may prevent the degradation or irreversible adsorption of bioactive product. Other media formulations may also be investigated (Carolissen-Mackay et al., 1997).

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