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Full Length Research Paper

Investigating the bacteriocin production profiles of isolated lactococcal strains: A comparative analysis

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Lactococcus lactis **strains present in wara (local cheese), and nunu (skimmed sour milk) were isolated. These were *L. lactis* WO81 and N13L. Nisin assays were by agar diffusion test and turbidimetric assay. There was no successful detection of an inhibition zone with wara. However, *L. lactis* strain WO81 from wara was able to produce nisin. Exponential growth occurred for about 14 h after some lag phase. The optimum incubation time of 2 to 6 h was established. Nisin concentration decreased with a subsequent increase in the incubation period. The effect of inoculum size was found not to be conclusive but directional to an optimal size of 1.0% (V/V). The suitable working temperature of 30°C and the pH value of 6 were discovered. Investigations into the phosphate sources revealed KH_2PO_4 as the best phos-phate source. A physiological link is proposed between these functions, growth and nisin concentra-tion. In the presence of sufficient glucose, growth was increased with the periodic addition of NaOH. Maximum nisin concentration was obtained when, at pH 6, the medium was buffered. In this study essentially it is noted that nisin has a lytic bactericidal mode of action.**

Key words: Local cheese, 'wara', 'nunu', nisin, bacteria, bacteriocin.

INTRODUCTION

Over two decades ago, Nettles and Barefoot (1993) reported several types of bacteriocins from food- associated lactic acid bacteria (LAB). Common among these are nisin, diplococcin and lactococcin (Ray et al., 2001; Fer-nandez et al., 2004; Corsetti et al., 2004), produced by *Lactococcus lactis*, a bacterium that occurs naturally in milk. Nisin has been the most extensively studied (Flores and Alegre, 2001). Nisin was the first bacteriocin derived from the fermentation of a lactic-acid bacterium and was approved by the FDA in April 1989 to prevent the growth of botulism spores in pasteurised processed-cheese sp-reads. Besides *Clostridium botulinum*, nisin inhibits a bro-ad spectrum of bacteria, which include *Listeria monocyto-genes*, *Staphylococcus aureus*, and *Bacillus cereus* (Bouttefroy et al. 2000; Jydegaard et al., 2000; Bizani and Brandelli, 2002; Risoen et al., 2004; Mataragas et al., 2002). Nisin is permitted up to various maximum pres-cribed levels in many countries of the world (Jack et al., 1995). The nisin is added exogenously, and allows manu-facturers to make a higher moisture product without risk of spoilage or health concerns for the consumers.

This could be incorporated into Nigerian nunu (skimmed sour milk) and wara (local cheese). Nisin offers processors a "clean" label as well as extending refrigerated shelf life by 14 to 30 days depending on the product (Morris, 1991). Ademuyiwa (1995) was able to isolate potential nisin-producing lactic acid bacteria from raw milk, nunu, and wara. A more advanced study on nisin-producing lactic acid bacteria seemed to be suitable and needed as a starting point for the delivery of safe nunu and wara to consumers, in line with today's microbiolo-gical safety rules for cottage industries.

Bacteriocins produced by isolates of *Lactobacillus* spp. from African fermented foods based on fermented maize (*Zea mays*), e.g ogi and cassava (*Manihot esculenta* Crantz), e.g. fufu have been successfully characterized (Olukoya et al., 1993; Ogunbanwo et al., 2003) with res-pect to their inhibitory spectrum. However, in these stu-dies and many more the microbial flora from nunu and wara were not characterised. Therefore, the objective of this study was to characterize nisin concentration among the isolates from nunu and wara. It was developed to include a study of the effects of inoculum size, incubation temperature, incubation time, pH, phosphorus source,

and the availability of NaOH, on the bactericidal activity of nisin.

MATERIALS AND METHODS

Preparation of wara: Various workers have described the preparation of wara (Aworh and Egunleti, 1985; Raheem, 2006). The art of making wara was adopted from the Fulani housewives in the Northern states of Nigeria. The locally made soft unripened cheese is made from unpasteurized milk and coagulated with the juice extracted from the leaves of *Calotropis procera* (Raheem 2006). Some of the starters involved in fermentation of cheese are *Streptococcus cremoris*, *Staphylococcus aureus*, *Lactobacillus casei* and *Micrococcus* species. The starter cultures are undefined multiple strains. Ogundiwin (1978) simulated the traditional procedures in the laboratory and reported that it took 65 minutes to complete cheese manufacture. He observed that coagulation was effected at temperatures between 65 and 68^{o}C at a pH range of 6.42 - 6.43. The processing time was between 30 - 35 min. The titratable acidity of most commercial cheese ranges from 0.2 - 0.27% and pH may be as low as 4.70. The increase in acidity is suggested due to post processing contamination by the lactic acid bacteria (Ogundiwin and Oke, 1983; Sanni et al., 1999).

Preparation of nunu: The Hausa women of Nigeria milk the cows, filling their long gourd containers. The raw cow milk according to Waters- Bayer (1985) was diluted by adding a mixture of water and kuka, a thickening agent made from the acid pith of baobab (*Andansonia digitata*) fruits constitute 'nunu' (skimmed sour milk). The pith of the kuka fruit is rich in vitamins B1 and C, can be mixed with water to serve as a refreshing drink, and is also used to treat intestinal disorders (von Maydell, 1983) . The water used in dilution of the raw milk came from shallow wells or streams and was not boiled before use. The rate of dilution varied greatly between wo-men. 'Nunu' is nutritious, refreshing and delicious when taken with 'fura' (millet powder), hence the popular 'fura de nunu' in the north-ern part of Nigeria. Variation in the local names ranges from fura de nunu, fura da nono, fura do nono. This Hausa delicacy abounds only during Ramadan (Moslem fasting month). Microorga-nisms that have been implicated in nunu include *Lactobacillus acidophilus*, *S. cremoris, Micrococcus luteus* and *L. lactis* (Akinyan-ju, 1989; Ademuyiwa, 1995). The starter cultures are undefined multiple strains since the fermentation generally depends on chan-ce inocula-tion.

Test organisms and cultivation media: *L. lactis* strains (WO81 and N13L) were used as nisin-producing microrganisms. *L. lactis* N13L was isolated from nunu and strain WO81 was isolated from wara in our laboratory and identified by their morphological and biochemical properties (Ademuyiwa, 1995). Before use, the nisin-producing *L. lactis* strains (WO81 and N13L) were activated in de Man-Rogosa-Sharpe (MRS) medium, commercial medium developed to support good growth of lactic acid bacteria, (de Man et al., 1960). Nisin mode of action was tested quantitatively with *Bacillus subtilis* MOO1995, which was obtained from the collection at the University of Ibadan, Ibadan, Nigeria, was used as a test organism in the bioassay. *B. subtilis* MOO1995 was activated in Tryptone soya broth by incubating these overnight at 30^{o}C. For the preparation of *L. lactic* cell-free supernatant (CFS), *L. lactis* strains were grown overnight at 30^{o}C in MRS broth. After growth, cells were removed by centrifugation (10,000 g, 10 min) and the supernatant was filter sterilized. It was used immediately or stored at –20^{o}C until used.

Assays of nisin in cultures of lactococci: Bacteriocin selective medium (BSM) with the following composition: 2 g/l beef extract, 10 g/l tryptone, 4 g/l yeast extract, 2 g/l glucose, 8.7 g/l $K_2HPO_4.3H_2O$, 8 g/l K_2HPO_4, 0.2 g/l $MgSO_4.7H_2O$, 0.05 g/l $MnSO_4$, 1 ml tween 80, 15 g agar, 1 g catalase, was prepared and cooled to about 50^{o}C. The suspension of the test organisms were diluted 1 in 10 with nor-mal saline solution and 2 ml of this dilution were added to each 100 ml of BSM at 48^{o}C with thorough mixing. The incubated med-ium was poured to a depth of 3 to 4 mm into flat-bottomed sterile Petri dishes and allowed to solidify. The plates were then inverted and stored at 4^{o}C for 1 h to facilitate the boring of wells. With the aid of a sterile cork borer (diameter = 1 cm) 3 wells were cut into the solidified medium and the disks so produced were removed and discarded. With a standard Pasteur pipette, the CFS was delivered in uniform quantities into the wells. The plates were covered and transferred carefully, without displacing the liquid in the wells, to a refrigerator (for the nisin to diffuse) for 12 h. Thereafter, the plates were transferred carefully to a 30^{o}C incubator and left overnight. The diameters of the zones of inhibition were measured to the nearest 0.1 mm by means of a bisector.

Determination of the modes of action of nisin: Nisin produced by *L. lactis* WO81 and N13L were tested by monitoring the growth of *B. subtilis* MOO1995 in CFS of *L. lactis*. The techniques of Tagg and McGiven (1971) and Benkerroum et al. (1993) were employed. Purified nisin and antibiotics such as streptomycin, ampicilin, azithromycin, nalidix acid, chlortetracycline (all from Difco), cefuroxin and cefriaxone (both from Oxoid) were subjected to the same test as positive control. The purified nisin was a commercial preparation (Nisapilin).

Optimisation and physiological studies: *L. lactis* present in wara (WO81), and nunu (N13L) were each grown for 5 h at 30^{o}C in 9 ml Bacteriocin Selective Medium (BSM) and subcultured for 10 h at 30^{o}C in 70ml BSM. A fresh culture of actively growing cells was always used as the inoculum. Fermentation was run in Erlenmeyer flasks (500 ml) containing 300 ml production medium. The fermenter was operated at 30^{o}C without aeration and slow agitation (50 rpm) was continuously provided to keep the fermentation broth homogenous. Samples of fermentation broth were withdrawn at 2 h intervals, and analysed for growth and nisin concentration to deduce the optimum incubation time. The centrifuged cells (10000, 10 min) were washed twice in saline (0.85% NaCl) (De Vuyst and Vandamme, 1992). The CFS served as nisin and assay was carried out by a turbidimetric method using *B. subtilis* MOO1995 (which is sensitive to nisin). Nisin concentration was measured as optical density (OD) at 660 nm using Beckman Du 7400 spectrophotometer. Bar charts of growth and nisin concentration against time for different sizes of inoculum were plotted. The inoculum sizes tested were 1% (v/v), 2% (v/v), 5% (v/v), 10% (v/v), and 15% (v/v), other-wise the inoculum size was maintained at 1% (v/v).

To establish the effect of different temperature regimens on growth and nisin concentration of the *L. lactis* strains, incubation was at different temperature regimens of 60, 50, 40, and 30^{o}C. The initial pH of BSM broth was adjusted to 6.0. The levels of pH tested were 4, 6, and 8 for OD and 2, 4, 6, 8, and 10 for nisin activity; otherwise the initial pH of BSM broth was adjusted to 6.0. BSM broth was adjusted using 0.1 N HCl or 0.1 N NaOH where appropriate. The effect of different phosphorus sources was tested on growth and nisin concentration of the test organism using BSM supplemented with 4% glucose (instead of the 2% glucose used in basal BSM) and 1% of an inorganic phosphorus source (replacing phosphorus present in the basal BSM). The varying phosphorus sources tested were KH_2PO_4 , K_2HPO_4, $(NH_4)_2HPO_4$, $NaHPO_4.2H_2O$, Na_2HPO_4, $Na_2HPO_4.H_20$, and $Na_2HPO_4.12H_20$ (all from BDH). The media were dispensed into cotton wool plunged into test tubes and sterilized by autoclaving at 121^{o} C for 15 min. On cooling, the tubes that were prepared in triplicate were inoculated aseptically with a fresh culture of actively growing cells. Neutralization of the lactic acid formed by the growing cultures was by the periodic addition of 10N-NaOH. Samples were checked for growth by comparison with the same experiment without the periodic addition of NaOH.

RESULTS

Two nisin-producing strains of *L. lactis* were isolated from local cheese (wara), and skimmed sour milk (nunu). Dur-

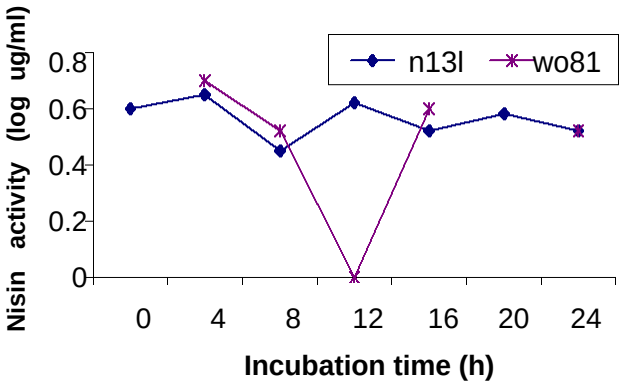

Figure 1. Effect of incubation time on nisin concentration (30^{o}C) of two *L. lactis* strains over a 24 h fermentation period.

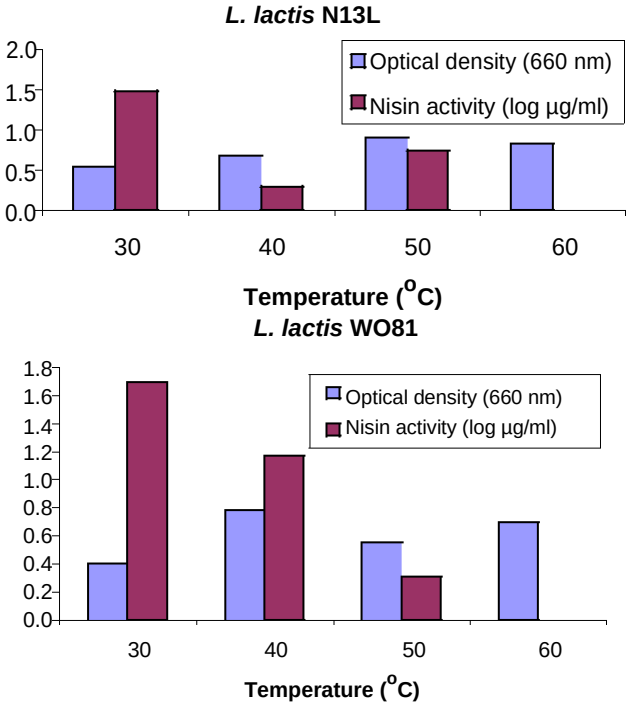

Figure 2. Effect of temperature regimen on growth and nisin production of *L. lactis* strains for 24 h in MRS broth at pH 6.0.

ing the demonstration of the bactericidal mode of action, the clearing around the well indicated the presence of nisin. A concentration of 0.48 µg nisin/ml was required to produce a measurable zone of inhibition (Figure 1). This is in accordance with Barry's (1986) report that the com-pound diffuses through the agar, setting up a concentra-tion gradient. The concentration is inversely proportional to the distance from the well (Figure 1). A zone of no gro-wth around the well indicates inhibition, which is the measure of nisin concentration.

For the agar diffusion method, no zones of inhibition were obtained when plates were incubated immediately after being set up, but when growth of the *B. subtilis* MOO1995 was delayed by refrigeration for 24 to 48 h to allow time for the nisin to diffuse, some inhibition was apparent after subsequent incubation.

The data in Figure 1 were based on 6 separate experi-ments and showed the sequence of change taking place during cultivation for 24 h in BSM. An incubation period of 2 to 6 h was the most favourable for nisin production for both strains tested. *L. lactis* WO81 had a value of 0.74log µg nisin/ml at 2 h when the incubation period was for 2 h. This was to be the highest nisin concentration reached in the strain during the time-course study. At 12 h, nisin concentration in this strain was 0.0log µg nisin/ml. Subse-quent incubation showed a decrease in nisin production. A gradual decrease was observed, probably due to a loss in bactericidal activity. Exponential growth occurred for about 14 h after some lag phase. Comparison between experiments showed that the length of the lag varied, the slope of growth curve varied, and the rate at which the nisin was formed during the experiment and subsequent-ly disappeared also varied. The small amount of nisin introduced with the inoculum at zero time could not be recovered after incubation for 2 to 4 h when exponential growth started.

Time-course studies were made on the effect of inocu-lum size of *B. subtilis* MOO1995 on the nisin concentra-tion by *L. lactis* strains N13L and W081 after 24 h at 30^{o}C. The observations (data not presented) showed that with increasing inoculum size of the sensitive indicator organism, there was a simultaneous increase in nisin concentration up to 1 ml inoculum and a subsequent dec-rease in nisin concentration, but the correlation broke down after 14 to 16 h of growth. The pH value decreased as lactic acid accumulated. The initial pH 6.0 decreased to a pH within a minimum of 4.4 and a maximum of 5.09 in 24 h; during the same time the O.D. reading of the lac-tococci increased from 0.13 to 0.78. During the expo-nential phase of growth, the O.D. doubling time ranged from 0.11 to 16. The greatest amount of nisin was pro-duced in the cultivation with the inoculum size of 1.0 ml.

The optimum temperatures for the production of nisin varied slightly with the two *L. lactis* strains examined (Fig-ure 2). The optimum temperature recorded was 30^{o}C. Nisin was detected at temperatures between 30 and 60^{o}C (Figure 2).

The cells of *L. lactis* WO81 reached O.D. of 0.18 after 8 h and had a bactericidal activity of 0.75log µg nisin/ml. Further incubation resulted in an increased O.D. but a rapid reduction in concentration.

Figure 3 shows the effect of pH on growth and nisin concentration. Both strains exhibited nisin production at all the pH levels. Growth and nisin concentration were optimal at pH 6 although at pH 4 greater nisin concen-trations was demonstrated by both strains from 4 to 6 h. Less nisin concentration tends to be better retain-ed at lower pH values.

The results are presented in Figure 4 of the influence of

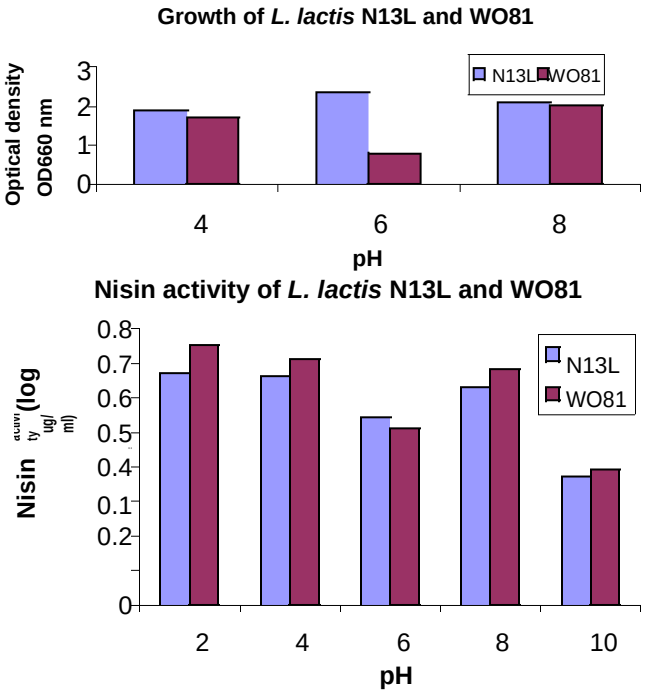

Figure 3. Effect of pH on growth and nisin production of *L. lactis* strains N13L and WO81 after 24 h at 30^{o}C in MRS broth.

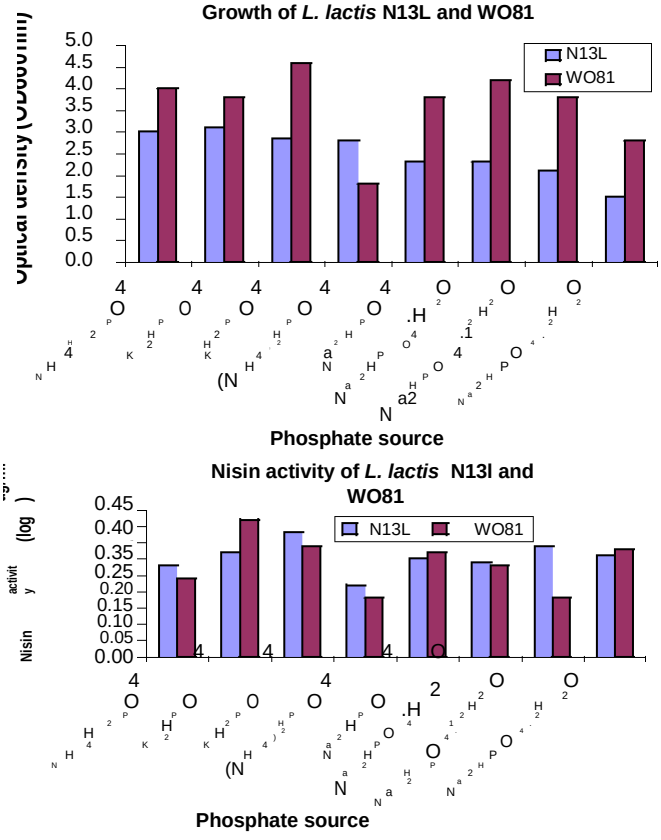

Figure 4. Effect of phosphorus source on growth and nisin production of *L. lactis* strains after 24 h at 30^{o}C.

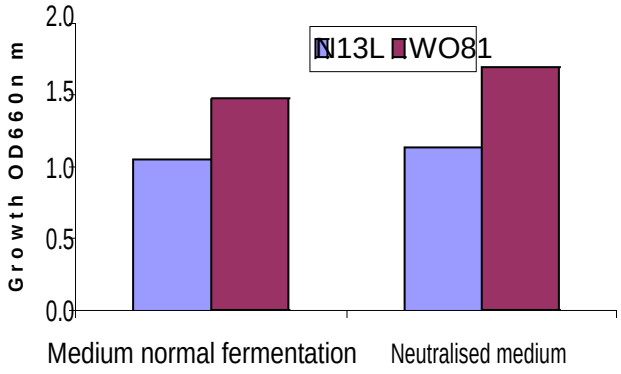

Figure 5. Effect of periodic addition of 10M – NaOH on growth and nisin concentration of *L. lactis* strains N13L and WO81 (Incubation time: 24 h).

different phosphorus sources tested. The two *L. lactis* strains showed an increase in growth by O.D. mea-surement with different phosphorus sources and the nisin concentration level was stimulated. In all, KH_2-PO $_4$ was found to be the best phosphorus source in that it gave the highest biomass and nisin concentra- tion levels. Growing cultures of *L. lactis* are sensitive to sodium hydroxide. Periodic additions of 10M- NaOH were found to improve the O.D. reading with time (ev-en with the BSM) by neutralizing of the acid formed (Figure 5).

DISCUSSION

There was no successful detection of an inhibition zone with wara, as reported in the work of Mocquot and Lefebvre (1956) who worked on a cylinder of cheese. It was possible that local cheeses examined were not made with a starter that produces nisin. This was disproved by the spot and streak method of Benkerroum et al. (1993) which, when employed, showed that the local cheese also had strain of *L. lactis* able to produce nisin, for an example, *L. lactis* strain WO81 (Ademuyiwa, 1995). The absence of inhibition may be because the diameter of the zone of inhibition produced is dependent not only upon the nisin concentration but also upon the amount of interfering substances present in the food extract. The difference in this research finding, compared to the report of Mocquot and Lefebvre (1956), may be because, unlike the wara that was used in this study, the cylinder of cheese had a smaller amount of interfering substances. According to Barry (1986), the size of the zone is dependent upon the rates of diffusion and cell growth.

The imperfect zone edge may be due to the unpurified quality of the nisin and also to the action of bacterial lysis, which cannot be exempted. When purified nisin and antibiotics such as streptomycin, ampicilin, azithromycin, naildix acid, chlortetracycline (all from Difco), cefuroxin and cefriaxone (both from Oxoid) were subjected to the same test, perfectly sharp zone edges were obtained

(Ademuyiwa, 1995).

In this study essentially it is noted that nisin has a lytic bactericidal mode of action. This is in line with the works of Bhunia et al. (1991) and Ahn and Stiles (1990) who reported that the extent of inhibition produced by each strain varies. During the late logarithmic period, there was apparent destruction of nisin. This may be due to the role of nisin in the bactericidal growth cycle.

The broke down in correlation after 14 to 16 h of growth may be due to the commonest source of error of variable distribution of bacteria in each inoculum size, especially in samples showing low optical densities. However, as propounded by Rayman and Hurst (1984) these differences were considered to be inherent in experi-ments done with different inocula and in experimental batches of media. This experiment showed that a maximum limiting population density (M'-concentration, Bail, 1929) was reached with this culture.

There may be a physiological link between temperature, growth, and nisin concentration. This may be due to the direct effect of temperature on the microbial growth rate. This result gives credence to the works of Delves-Broughton (1990) and Van den Berghe et al. (2006), who reported optimum temperatures of 37 and 25^{O} C for nisin production respectively. Similarly, Leroy and de Vuyst, (1999) reported that bacteriocin activity were very much influenced by changes in temperature and pH.

From this study it was found that pH is a controlling factor for nisin concentration. The results of Yang and Ray (1994) also identified that growing bacterial cells at optimum pH can increase the concentration of a bacteriocin in a simple medium. Nisin concentration was higher in the acidic range.The highest bactericidal activit obtained was at pH 2 to 4. This is in accordance therefore with the report of Bernard and Ferda (1991) that nisin is neither very active nor stable at high pH values. The optimum pH (6) for growth of the isolated strains affected their nisin concentration. There was a decrease in nisin concentration on either side of the optimum pH 6 and this agreed with the views of Liu and Hansen (1990), which state that nisin is most stable at pH 2 and its concentration decreases drastically at basic pH values between 8 and 9. Findings from this study clearly indicated that cell yield and nisin concentration levels are strongly stimulated by added phosphorus, irrespective of the type of phosphorus source. Additionally, Leroy and de Vuyst (1999) reported that the concentration of biomass was closely related to bacteriocin activity, indicating primary metabolite kinetics, but was not the only factor of importance. In agreement with the findings of De Vuyst and Vandamme (1993), KH_2PO_4 was found to be the best phosphorus source for nisin concentration. It clearly demonstrates the potential of this mineral to specifically support the growth of lactic acid bacteria. Considering the positive correlation between biomass and nisin concentration levels (De Vuyst and Vandamme, 1991), the results in this study suggested that inorganic phosphate stimulated nisin concentration.

Periodic additions of 10M-NaOH may improve optical density O.D reading due to the effect of neutralizing the acid formed when the nutrient did not become a limiting factor. Rogers and Whittier (1928) obtained similar results showing that when 10M-NaOH was periodically added to growing cultures of lactococci, considerable increase in growth was obtained. According to the fermentation profiles presented by Flores and Alegre (2001), the addition of 4M-NaOH has been demonstrated to reduce the lactic acid produced during fermentation and subsequently enhancing concentration of nisin. Matsusaki et al. (1996) also reported fermentation system with pH control via addition of NaOH.

Although nunu and wara are popular traditional milk products in Nigeria, there shelf lives are less than two days. In these milk products lactic acid bacteria were identified as the major part of the fermentation flora. In addition to the anti-microbially active organic acid bacteria produce bacterocins, which can reduce the growth of spoilage organisms and improve the hygienic condition of the product (Lindgren and Dobrogosz, 1990; Olukoya et al., 1993; O'Sullivan et al., 2002), thus reducing the safety hazard the consumers are subjected to. As the spoilage bacteria compete with one another for the highly nutritious nunu and wara, bacteriocin-producing bacteria may make the prevailing condition unsuitable for food borne pathogens and spoilage organisms. Hence its potential uses as biological food preservative. Specifically, nisin is antagonistic to spore-formers (Hurst, 1972).

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