Defence Life Science Journal, Vol. 2, No. 2, April 2017, pp. 212-215, DOI : 10.14429/dlsj.2.11358

RESEARCH PAPER

Turbidimetric Assay of Nisin in Tender Coconut Water

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ABSTRACT

Nisin activity in tender coconut water using Lactococcus lactis subspecies cremoris as test microorganism was estimated using turbidimetry by measuring optical density at 600 nm after 6 h. of incubation at 37 °C. The test microorganism was cultivated in nutrient broth with different concentration of nisin along with tender coconut water. A standard curve was drawn between optical density and known concentration of nisin. The nisin concentration of the test samples was measured by curve fitting method using the optical density values. The results thus obtained by this method were reproducible, rapid and more accurate in tender coconut water and hence it may be employed for the determination of nisin activity in tender coconut water on regular basis for quality control purposes.

Keywords: Nisin; Tender Coconut water; Turbidimetric assay

1. INTRODUCTION

India accounts for 11,930,000 tons of the world's coconut production and is one of the major players in the world's coconut trade. As a result of economic globalisation, efforts have been made to popularise Indian coconut products accessible to consumers across the world. Emphasis has been given for the development of value added products through newer processing technologies. Tender coconut water, the liquid endosperm is the most nutritious wholesome organic food/ natural drink and has great potential as a health drink in India and international market. In its natural state, it is sterile and used as oral rehydration medium for children suffering from gastroenteritis. Tender coconuts can not be kept in its natural form for a longer period due to the onset of undesirable quality changes[1]. Further, its bulkiness and weight of the coconuts pose the problem of handling and transportation. Hence, development of suitable simple preservation technique is important to retain the original quality characteristics with extended shelf life in a convenient package. Though sterilisation tender coconut water in packed in pouch/can will address the problems, the final product will be devoid of unique and delicate tender coconut flavour attributed by the presence of a group of delta lactones which is thermally unstable above 100 °C. In the absence of suitable preservative, the product pasteurised below 100 °C was highly susceptible for deterioration and spoilage[2].

The use of nisin in tender coconut water among various chemical preservatives in combination with pasteurisation at 96 °C reduced the severity of heat treatment while protecting from gram-positive vegetative bacteria and particularly bacterial spore-formers[2]. Accordingly, a combination preservation technique consisting of addition of nisin and heating has been standardised[3].

The type a lantibiotic nisin produced by several *Lactococcus lactis* strains, and one *Streptococcus uberis* strainis a small antimicrobial peptide that inhibits the growth of a wide range of gram-positive bacteria, such as *Bacillus*, *Clostridium*, *Listeria* and *Staphylococcus* species[4]. It is nontoxic to humans and used as a food preservative (E234) in more than 50 countries including the EU, the USA, and China. India has permitted the use of nisin in tender coconut water up to 10,000 ppm. However, national legislations concerning maximum addition levels of nisin in different foods vary greatly. The addition of nisin in meat sausage was studied for its efficacy in improving storage stability after mild treatment[5]**.** The study of assay of nisin in foods for its efficacy has become important in commercial production[6]. Therefore, there is a demand for non-laborious and sensitive methods to identify and quantify nisin reliably from different food matrices.

Different assay methods are used to assess the bactericidal activity include

(i) Spotting culture supernatants on indicator lawns
(ii) Cross-streaking bacteria
(iii) Overlapping colonies of the producer strain with an indicator lawn
(iv) Agar well diffusion of culture supernatant and
(v) The flip plate method[7].

Hakovirta[8], *et al.* studied the availability of nisin by using bioassay method in milk, processed cheese, salad dressings, canned tomatoes, and liquid egg. The horizontal inhibition assay, based on the inhibitory effect of nisin to *Micrococcus*

Received : 20 March 2017, Revised : 04 April 2017
Accepted : 15 April 2017, Online published : 12 May 2017

luteus is the base for most quantification methods developed so far. However, the sensitivity and accuracy of the agar plate diffusion method is affected by several parameters. However, modified of agar plate diffusion assays are the most widely used methods even though the limitations of such assays are generally recognised[9]. The quantitative estimation of a bacteriocin is based on the critical dilution of antagonistic activity[10]. An enzyme-linked immunosorbent assay (ELISA) using polyclonal antiserum for nisin detection was used with commercial cheese samples[11]. This method had a limit of detection of 1.9 X 10^{-2} IU per ml and yielded results that correlated well with bioassay results.

Berridge and Barret[12] developed a rapid method for the turbidimetric assay of antibiotics. However, Pitkin[13], *et al*. developed a quantitative semi-automated turbidimetric bioassay for cefazaflur, using *Streptococcus faecium* as the indicator. In this study, the dose response line was plotted point to point using the natural log of the absorbance versus natural log of the concentration. This assay is reported both accurate and precise and is more rapid than traditional plate assays for antibiotics. Further attempts are made to develop a quantitative method using automated turbidometry to assess the antimicrobial efficacy of bacteriocin-like inhibitors produced by *pseudomonas aeruginosa, pediococus dammosus* and *pediococcus pentosaccus* and the growth of the test strain namely *pseudomonas aeruginosa* was kinetically monitored and various growth curve parameters were used as quantitative indicators of inhibition[14]. Flores[15], *et al*. reported a modified turbidimetric assay method for nisin in total incubation period of approximately 6 h. using *Lactococcus lactis* subsp. c*remoris* as test microorganism. In this method, some steps such as over night culturing of test organism and series of dilution of fresh medium followed by incubation used in the Hurst method were eliminated. However, these modified turbidometry assay methods have not been evaluated to assess their antimicrobial efficacy of the nisin in the products.

In this study, attempt was made to assess the antimicrobial efficacy of nisin in tender coconut water using turbidimetric assay method with suitable modification for quality control purposes as an alternate for agar diffusion assay method.

2. MATERIALS AND METHODS

Nisin (2.5%) was obtained in the form of Nisaplin™ (M/s Aplin & Barrett Ltd., UK) with an activity of 1.0X10^6 IU $g^{-1}$and stored at 4°C. *Lactococcus lactis* subsp. *Cremoris* was used as test microorganism. The test microorganism was cultured in Erlenmeyer's flasks with 300 ml of medium with the following composition (% w/v): meat extract, 1; yeast extract, 1; tryptone, 1; glucose, 1; NaCl, 0.5; Na_2HPO_4, 0.2; $MgSO_4.7H_2O$, 0.02; pH 6.8±0.2. After fermentation, the medium was distributed in deep tubes containing glycerol (8.5 ml of cultured medium and 1.5 ml of glycerol) and stored frozen. The tubes were used as Inoculum for subsequent assays. To obtain growth curve of test microorganism, the samples were collected at each hour and their optical density (OD) were measured spectrophotometrically at 600 nm (Model Spectronic: Genesys 2).

2.1 Nisin Standard

0.05 g of nisin (10^6 IU/g) was weighed accurately and dissolved in 40 ml 0.02 N hydrochloric acid (HCl) and kept at room temperature for 2 h. The solution was further diluted in order to obtain a standard concentration in the range of 1.0 µg/ml to 50.0 µg/ml. A fresh standard nisin solution was prepared and used every day.

2.2 Bioassay of Nisin

One tube of the test microorganism pre-cultured was diluted with 10ml of sterilised enriched nutrient broth with the following composition (w/v): meat extract, 1; yeast extract, 1; tryptone, 1; glucose, 1; NaCl, 0.5; Na_2HPO_4, 0.2; $MgSO_4.7H_2O$, 0.02; pH 6.8±0.2. One ml of this culture (6.0 x 10^2 cfu/ml) was distributed in deep tubes containing 8 ml of this same basic free medium and 1 ml of standard nisin solution containing different concentrations. An another set of nisin standard solution was also prepared by using tender coconut water instead of using 0.02 N hydrochloric acid (HCl) to avoid the interference of other constituents of food during product analysis. In the control tube the standard nisin solution was replaced by 1 ml of medium. All tubes were incubated at 30°±2 °C without shaking for six hour and their OD were measured at 600 nm. The growth was stopped by injecting into each tube 1 ml of 0.004 per cent of thiomersalate solution. A series of standards were setup in triplicate for each assay. A standard curve was obtained by plotting OD versus concentration of nisin. Concurrently, estimation of nisin was also carried out by well diffusion method for comparison purposes.

2.3 Extraction of Nisin from Coconut Water

The unknown concentration of nisin was extracted from three different commercial samples of tender coconut water as described by Tramer and Fowler[17] and bioassay was carried out as described above.

3. RESULTS AND DISCUSSION

The growth of the test organism namely *Lactococcus lactis* subsp. *Cremoris* was observed up to ten hours and the OD of the collected samples was measured at each hour. The OD was also taken for test samples of tender coconut water. The nisin concentrations were calculated using standard graphs after applying proper dilution factor. Accordingly, the growth curve obtained by plotting time versus OD_{600} is shown in Fig. 1. The mean values of three independent measurements at OD_{600} were taken to estimate the growth inhibition of test microorganism against control. The lag phase of the growth of the test microorganism was two hours and reached the stationary phase in six hours of incubation and thus it was defined as the incubation time to draw the standard curve of nisin as well as to determine nisin concentration in other solutions. Flores[6], *et al* have reported that six hours of incubation was adequate for the assay of nisin using modified turbidimetric method. In our experiment also the incubation time is found to be the same and confirming that six hours incubation time is essentially needed for the turbidimetric assay of nisin in the product such as tender coconut water.

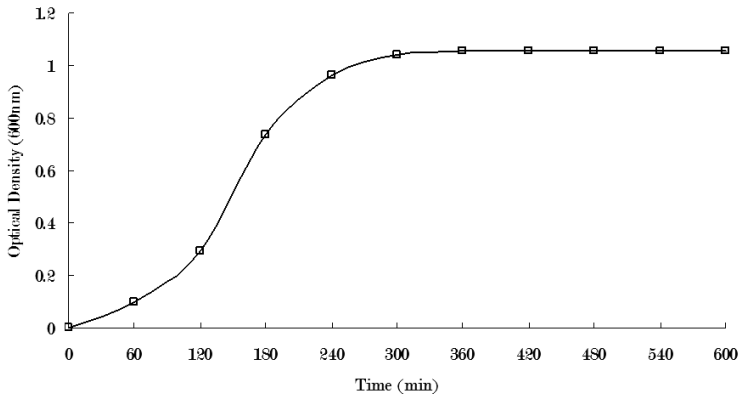

Figure 1. Growth curve of lactococcus lactis (cremoris).

The quantitative activities of nisin in standard solutions were determined in liquid substrate by turbidimetric method. Concentrations of nisin ranges from 1-50 µg/ml were plotted against average optical density of triplicate readings for standard nisin solution in 0.02N HCl and tender coconut water extract (Fig. 2). The growth pattern of microorganism with respect to concentration of nisin showed a sigmoid curve in both the cases of nisin standard solutions. The accuracy and efficacy of method is tested by the addition of known quantity of nisin at 3 mg/l, 4 mg/l, 5 mg/l in tender coconut water. A similar growth pattern for *Lactococcus lactis* (*cremoris*) was reported by Turcotte[17], *et al*. Further, it is also observed that there is an inverse relationship between concentration of nisin and the population of test microorganism indicating that there is no significance effect of interfering substance on the pattern of the microbial growth.

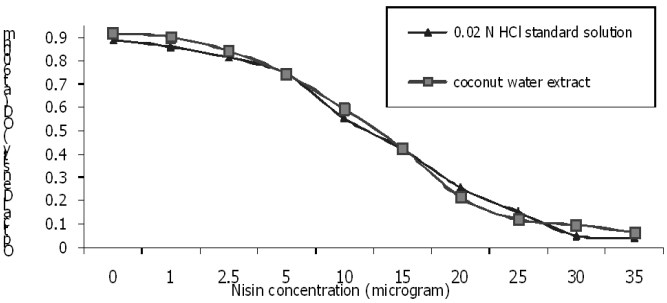

Figure 2. Nisin activity of standard solutions in 0.02N HCI V/S with tender coconut water extract solution.

The estimated concentrations of nisin in tender coconut water are shown in Table 1. The nisin concentration in tender coconut water calculated by using graph found to be more accurate and found to be comparable with the well diffusion method. Further, the curve of the standard nisin in HCl is similar to tender coconut extract and it indicates that turbidimetric assay method may be adopted for the direct estimation of nisin other products such as juices, cordials, etc., as in the case of tender coconut water.

Table 1. Nisin in tender coconut water obtained by two different standard solutions

Added concentration of nisin (mg/l)	Estimated concentration of nisin (mg/l) extracted from	
	0.02 N HCl	Tender coconut water
3.0	2.75	2.80
4.0	3.50	3.75
5.0	4.65	4.80

4. CONCLUSION

The preparation of nisin solution as well as initial population of microorganism is found to be critical for the accuracy of the turbidimetric assay method. The results obtained by this method may be directly correlated with the actual quantity of nisin in tender coconut water. This method can be used as alternative to agar diffusion assay, which is most widely used in routine measurement of nisin activity. The turbidimetric assay method is simple and reproducible and no diffusion related problems. Further, this method may also be employed for the products which have low concentration of nisin. This method may be adopted in tender coconut water processing plants for the routine analysis of nisin concentration in tender coconut water for quality control.

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He has contributed to the study by guiding for the experimental study and writing of the paper.