Research Journal of Biological Sciences 5 (11): 727-730, 2010
ISSN: 1815-8846

Recovery of Bacteriocin (NISIN) from *Lactococcus lactis* and Testing its Ability to Increase the Shelf Life of Vegetables (Carrot and Beans)

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Abstract: The ability of both *Lactococcus lactis* and the extracted bacteriocin against food spoilage bacteria and their usage as biopreservative were investigated in this study. *L. lactis* was isolated from the cow milk and the bacteriocin produced by *L. lactis*, namely Nisin was extracted. *L. lactis* and the extracted bacteriocin were used to preserve vegetables (carrots and beans). The vegetables were treated with both *L. lactis* and the extracted bacteriocin then observed for the spoilage. The untreated vegetables were found to be spoiled by both bacteria and fungi within 5 days but only 15 days of observation, the fungal invention was seen on the skin of the vegetables treated with *L. lactis* and extracted bacteriocin. In the present study, *L. lactis* and the extracted bacteriocin were found to enhance the shelf life of vegetables for >15-25 days depending on concentration without the use of refrigeration. It can be concluded that the biopreservatives used in the study can effectively replace the use of refrigeration thus minimizing the release of CFC to the atmosphere. The biopreservation with *L. lactis* and bacteriocin is also cost effective and it could possibly enhance the health of consumers as *L. lactis* belongs to probiotic group of LAB.

Key words: *L. lactis*, bacteriocin, biopreservation, carrots and beans, atmosphere, refrigeration

INTRODUCTION

Artificial chemical preservatives are being employed to limit, the number of microorganisms capable of growing within foods but increasing consumer awareness and potential health risks associated with some of these substances have initiated, the researchers to examine the possibility of using bacteriocins produced by LAB as biopreservative (Abee *et al*., 1995).

LAB are known to produce many different antibacterial substances including bacteriocins which can inhibit the growth of several undesirable gram-positive bacteria of the genera Bacillus, Enterococcus, Listeria, Clostridium and Staphylococcus. Many bacteriocins have been isolated and there is an increasing interest in using these bacteriocins as natural food preservatives (Noonpakdee *et al*., 2003). In 1996, Stiles has described that biopreservation is the extension of storage life and enhancement of food safety using the natural or controlled microflora or their antimicrobial products (Rodgers, 2001).

Bacteriocins are ribosomally synthesized, extracellularly released, low molecular weight peptides or proteins (usually 30-60 amino acids) which have a bactericidal or bacteriostatic effect on other bacteria of the same species or across genera (Klaenhammer, 1988; Abee *et al*., 1995; Chen and Hoover, 2003; Senthilkumar and Pandian, 2009). One of the most industrially relevant bacteriocins is Nisin which is produced by *L. lactis* belonging to LAB (Hurst, 1981).

The lactococcal bacteriocin named Nisin (or group N inhibitory substance) was 1st marketed in England in 1953 and since then has been approved for use in >48 countries. The successful development of Nisin from an initial biological observation through regulatory approval to commercial application is a model that has stimulated significant resurgence in bacteriocin research in recent years (Deegan *et al*., 2006).

MATERIALS AND METHODS

L. lactis was isolated from cow (Jersey bread) by using MRS agar medium. The pathogenic microbes were isolated by using EMB agar medium, KB agar medium and SS agar medium. The isolated *L. lactis* was inoculated in buffered TGE broth (0.5% sodium citrate, 0.1% sodium acetate and 0.05% dipotassium phosphate-pH 6.5) and incubated at 30°C for 2 days. After incubation, pH was adjusted to 2.5, using 5% phosphoric acid (Yang *et al*., 1992) and heated at 75°C for 15 min. Then centrifuged at 3,000 rpm for 20 min, supernatant (crude extracts) were collected which contains the bacteriocin (Senthilkumar and Pandian, 2009). The recovered bacteriocin was purified by salt precipitation method

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(0.4 g mL^{-1} of ammonium sulphate salt) and dialysis. The amount of protein was then estimated by Lowry's method. The efficiency of the purified bacteriocin against the isolated spoilage bacteria was tested by Agar well diffusion assay. *L. lactis* and bacteriocin were applied over vegetables (carrot and beans), under sterile conditions. An untreated control group of vegetables was maintained for the comparison.

RESULTS AND DISCUSSION

Pale white colonies were identified as *L. lactis* from the results obtained. They were gram-positive rods, salt tolerant in 4% NaCl containing medium and arginine hydrolyzed. Four isolates were selected and the bacteriocin were extracted and purified. The bacteriocins were named as Nis I-IV. They were found to contain 300-400 μg mL^{-1} of protein where, BSA was used as standard protein. The efficiency of extracted bacteriocin against spoilage bacteria were tested by Agar well diffusion assay. The results were shown in Table 1 and Fig. 1.

Biopreservation of vegetables: The treated carrots were found to be preserved for >15 days where as untreated carrots (control) were found to be normal for 7 days only.

Table 1: Agar well diffusion assay

	Zone of inhibition (mm)			
Tested bacteria	Nis I	Nis II	Nis III	Nis IV
Bacillus sp.	7 (R)	8 (R)	9 (R)	12 (S)
E. coli	7 (R)	8 (R)	9 (R)	12 (S)
Enterobacter sp.	10 (R)	12 (S)	14 (S)	21 (S)
Klebsiella sp.	8 (R)	10 (R)	12 (S)	19 (S)
Pseudomonas sp.	*	*	*	*
Salmonella sp.	**	**	**	**

*Pigment lysis with no zone; **No zone; R: Resistant with zone of inhibition <12 nm; S: Sensitive with zone of inhibition >12 nm

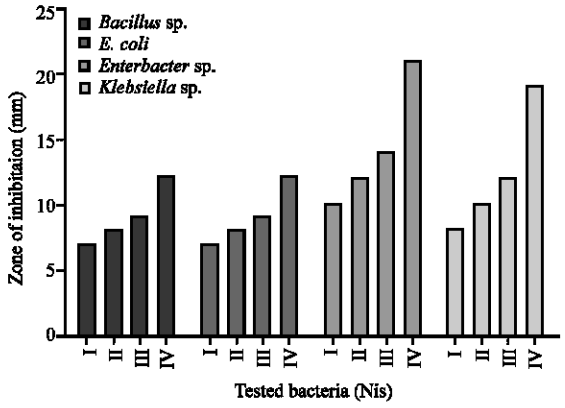

Fig. 1: Measurement of zone if inhibition of the extracted bacteriocin against the tested bacteria

The data on biopreservation of carrots was shown in Table 2 and Fig. 2. The treated beans were found to be preserved for >8 days while untreated beans (control) were found to be normal for 7 days only. The data on biopreservation of beans was shown in the Table 3. The cells of *Lactococcus* sp., extends into a chain which makes them difficult to differentiate from *Lactobacillus* sp. in gram staining.

The differentiation between the genus *Enterococcus* sp. and *Lactococcus* sp. is difficult on a morphological basis. Salt tolerance at different temperature was observed in this study to differentiate both the genus. *L. lactis* was grown in 4% NaCl MRS medium but not in 6.5% NaCl MRS medium. This shows that *L. lactis* is not salt tolerant whereas, *Enterococcus* sp. can grow at 6.5% NaCl MRS medium. *L. lactis* grow at a temperature of 10°C but not at 45°C (Samarzija *et al*., 2001). But *Enterococcus* sp. was grown at both the temperatures. *L. lactis* is lactose fermentative but *Enterococcus* sp. is not lactose fermentative.

Table 2: Data on biopreservation efficiency of carrots

Biopreservative used	No. of days preserved/found intact	Contagion after specific time period
Carrots control	7	5th day, bacterial rot and fungus on skin
Lac	16	11th day, fungus on skin
Nis I	15	12th day, fungus on skin
Nis II	22	19th day, fungus on skin
Nis III	23	17th day, fungus on skin
Nis IV	28	26th day, fungus on skin

Table 3: Data on biopreservation efficiency of the treatments on beans

Biopreservative used	No. of days preserved/found intact	Nature of contamination after specific time period	Observed colour after due time
Beans control	4	Fungal	Green
Lac	9	-	Yellow
Nis I	7	-	Yellow
Nis II	5	Fungal	Yellow
Nis III	8	-	Yellow
Nis IV	8	Fungal	Yellow

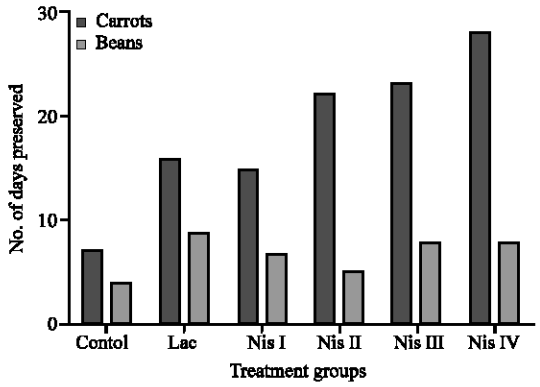

Fig. 2: No. of days of preservation of carrots and beans using exracted bacteriocins

In the present study, the bacteriocins extracted using the TGE medium were found to contain protein at the concentration of 300-400 μg mL^{-1} which is same as compared with the study by Senthilkumar and Pandian (2009). The effect of extracted bacteriocins against the isolated spoilage bacteria were tested by Agar well diffusion assay.

The results showed that Nis III (350 μg mL^{-1}) and IV (400 μg mL^{-1}) were found inhibiting spoilage bacteria effectively when compared with Nis I (300 μg mL^{-1}) and II (350 μg mL^{-1}). Senthilkumar and Pandian (2009) has tested the antimicrobial activity of Nisin at the concentration of 400 μg mL^{-1} against spoilage bacteria and found it effective.

The untreated vegetables were found infected by both bacteria and fungi within 7 days. The results showed that *L. lactis* treated were found uninfected for 15 days whereas the nisin coated were uninfected for 15-25 days depends on the concentration. Nisin with concentration of 400 μg mL^{-1} were effective in preventing microbial spoilage of vegetables than at the concentration of 300 and 350 μg mL^{-1} of Nisin. From this, it was concluded that the Nisin was effective in controlling the spoilage bacteria at the concentration of 400 μg mL^{-1}. The same concentration was found effective in preservation of pasteurized eggs for a period of 20 days (Senthilkumar and Pandian, 2009).

In case of preservation of the carrots, the samples were found to be uninfected with microbes for >15 days and beans for >8 days. The vegetables were treated only with *L. lactis* culture and the extracted bacteriocin. Whereas in previous studies by Ukuku *et al.* (2009) and Ukuku and Fett (2004), the Nisin has been used in combination with EDTA, sodium lactate and potassium sorbate to reduce the spoilage bacteria on the food. Since, EDTA is carcinogenic and the other compounds were chemicals, the present study was done only with *L. lactis* culture and the extracted bacteriocin. Nisin incorporated package films were used to store beef carcasses for a period of 20 days in refrigeration condition (Chen and Hoover, 2003).

The treated vegetables used in the study were stored in airtight containers at room temperature and found preserved for >15-25 days depends on the concentration of Nisin. This would reduce the usage of refrigerator and hence, reduce the release of CFC. Now-a-days, consumer awareness for the harmful effects of the use of chemical preservatives is gaining importance. One of the effective alternative for this is biopreservatives. According to Bernbom *et al.* (2006), the use of *Lactococcus* sp. and Nisin is not causing any harm to the human intestinal micro biota. The combination of growth factor delivery with a probiotic approach may offer possibilities for formulating dietary supplements for children during their weaning transition stage (Cheung *et al.*, 2009). Hence, it can be hypothesized that the use of *Lactococcus* sp. and Nisin as a food preservative can effectively replace the use of chemical preservatives and can prevent its harmful effects.

CONCLUSION

The study was carried out in order to increase the shelf life of vegetables using *L. lactis* and Nisin. The results were showing favour to use *L. lactis* and Nisin as biopreservative. The freshness, colour, texture and nature of the vegetables did not change by the use of *L. lactis* and Nisin. On an average till day 15, the vegetables were found to be not affected by microorganisms, upon treatment with *L. lactis* and Nisin. The vegetables were found infected by fungus since, the *L. lactis* and Nisin does not have antifungal property. Bacterial rot was not noticed in the *L. lactis* and Nisin treated vegetables. Thus, it was concluded that the *L. lactis* and Nisin can be used to increase shelf life of vegetables upto an average of 15-20 days without refrigeration.

Since, the chemical preservation methods and the refrigeration procedures were found to be disadvantageous with respect to alteration in food nutritive value, increased cost and high release of CFC contributing to environmental pollution. Thus, it can be summarized from this study that the use of *L. lactis* and Nisin is an eco-friendly, natural, cost effective and beneficial method of preserving vegetables.

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