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Control of rope spore forming bacteria using carambola (*Averrhoa carambola)* fruit pomace powder in wheat bread preparation

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RESEARCH ARTICLE

Abstract

Wheat bread is a high moisture, low acid product, susceptible to spoilage by *Bacillus* spp., as the endospores survive the baking temperatures. Carambola (*Averrhoa carambola)* fruit pomace, a by-product obtained after juice extraction, has an insoluble dietary fibre content of 61.0±1.2% and is rich in antimicrobial phenolic compounds of 17.8±1.1 gallic acid equivalent/g (water extract). The anti-nutritional content of oxalic acid in the fruit is reduced to 0.15 mg/g in the pomace. Studies on the antimicrobial activity of the dried pomace powder in dimethyl-sulfoxide as determined by the disc diffusion assay showed that the Gram-positive bacteria tested were more sensitive than Gram-negative bacteria. The minimum inhibitory and minimum bactericidal concentration of *Bacillus cereus* and of *Bacillus subtilis* was 1.25 mg/ml of the dry pomace powder. Incorporation of carambola pomace powder into wheat flour for bread preparation at a 5% level resulted in a bread of acceptable sensory profile with a high desirable fibre content. The rope spore count of the bread with carambola fruit pomace powder at this level showed a count of 0.7 $\log_{10}$/g, an inhibition equal to that of bread with chemical preservatives added while the bread with no preservatives had a count of 8.76 $\log_{10}$/g at the end of 72 h when incubated at 35 °C, resulting in a product that was safe with added high fibre a desired nutrient quality.

Keywords: antimicrobial activity, *Bacillus*, bread, carambola fruit pomace, rope spore count

1. Introduction

Averrhoa carambola L., commonly known as star fruit or carambola, is grown extensively in India and China (Hayes, 1960). Fruits are oblong, longitudinally 5- to 6-angled, about 6-15 cm long and up to 9 cm in diameter. They have a thin, waxy, orange-yellow skin and when fully ripe the flesh is yellow and juicy. The leaves and fruits of this plant have been used in folk medicine, as an appetite stimulant, a diuretic, an antidiarrhoeal, and a febrifugal agent, as well as in the treatment of eczemas (Moresco *et al.*, 2012). The fruit has also been shown to have hypocholesterolemic and hypoglycemic properties (Chau *et al.*, 2004a). Carambola fruits are rich in dietary fibre, especially insoluble fibre and contain a high concentration of water-insoluble fibre-rich fraction (50.8 g/100 g of pomace dry weight) (Anonymous, 2004). The by-product pomace obtained after juice production is promising as a functional food fibre.

The wheat bread industry in India is a 4 million tonnes industry, growing at the rate of 6% and is expected to grow at the same rate in the future. Bread is finding popularity as an item consumed for breakfast in place of traditional products and hence bread production has been on the rise. The industry consists of organised and unorganised sectors, contributing around 45 and 55% of the total bread production, respectively (www.aibma.com). Bread is a high moisture and low-acid product with a water activity of 0.96 to 0.98 and a pH of more than 5.6 (Smith, 1993). These intrinsic properties if combined with other favourable factors of storage temperature, relative humidity, and packaging material make it susceptible to both bacterial

and mould spoilage. The temperature reached during the baking process ensures the inactivation of the non-spore forming bacteria. However members of the genus *Bacillus,* like *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus cereus,* that can form highly resistant dormant endospores in response to nutrient deprivation and other environmental stresses (Nicholson *et al.*, 2000) survive the baking process. These bacterial spores are naturally present in the raw material, i.e. wheat flour (Pepe *et al.*, 2003). Many of these species, particularly *B. subtilis,* cause rope spoilage in bread characterised by discoloured, soft and sticky internal crumb and an unpleasant sweet, fruity odour (Kirschner and Holy, 1989; Voysey, 1989). Traditionally, the most practical, common and cost effective approach adopted by the baking industry is the use of chemical preservatives incorporated directly into the product (Rosenkvist and Hansen, 1995; Sockett, 1991;Todd, 1982). Propionic acid and calcium propionate are usually employed at concentrations of 0.1 and 0.2%, respectively (Seiler, 1994). However, in recent years the use of chemical preservatives is discouraged. In this manuscript, a novel approach of incorporating the fruit pomace of carambola into the wheat flour before baking is a step in the direction of use of natural ingredients to control bacterial spoilage. It was found that the growth of *B. subtilis*, the causative agent of ropy bread was controlled while at the same time increasing the dietary fibre of the baked bread.

2. Materials and methods

Preparation of carambola pomace powder

The yellow carambola ripe fruits procured from the local market were washed in water and air dried. The fruits were then crushed through a pulper of 30 mesh sieve (Sen Berry Co. Ltd. New Delhi, India), pressed with hydraulic press to separate juice and pomace. Fresh pomace was washed 2-3 times in water and then dried in a hot air drier at ~55 °C, till the residual moisture content reached ~5%.The dried pomace was powdered using an Apex mill (Cadmach machinery Co. Ltd., Gujarat, India) and the powder (~1 mm) obtained was stored at room temperature in polypropylene bags till use.

Characterisation of the dried carambola pomace powder

Determination of total dietary fibre, soluble dietary fibre and insoluble dietary fibre was carried out according to AOAC official method 991.43 (AOAC, 2000). Bulk density, water holding capacity and oil holding capacity of carambola pomace was determined according to Sudha *et al.* (2007). The titrable acidity of the fresh pomace was measured and expressed as % citric acid by titration method (Ranganna, 1986). Oxalic acid content was determined according to Kafkas *et al.* (2006). Starch was estimated according to Hassid and Neufeld (1964).

The total sugars were determined according to Dubois *et al.* (1956). To the water extract of pomace (1 ml), 0.6 ml phenol (5%) was added, followed by 3.6 ml of concentrated sulphuric acid along the sides of the tube. A yellowish orange colour was developed, which was read at 490 nm using distilled water as blank. The reducing sugars were determined according to Miller (1959). To the water extract of pomace (1 ml), di-nitro salicylic acid reagent (1 ml) was added and kept in boiling water bath for 10 min. Distilled water (4 ml) was added to each tube and cooled. The yellowish red colour developed was read at 540 nm. Distilled water served as blank. All analyses of the samples were carried out in triplicates.

In vitro antimicrobial activity

Preparation of inocula

Preliminary studies on the antimicrobial activity of the carambola pomace powder were carried out by the disc diffusion assay as outlined by Bauer *et al.* (1966). A suspension of the dried carambola pomace powder, 125 mg/ml was prepared in dimethyl-sulfoxide (DMSO). The microorganisms used in the assay were *Escherichia coli* serovar 01:K1:H7 (ATTC-11775), *Salmonella enterica* subsp. *enterica* serovar Typhimurium (ATTC-25241), *Listeria monocytogenes* (ATCC 13932), *Staphylococcus aureus* (ATTC-12600), *B. cereus* (ATTC-11778), *B. subtilis spizizenii* (ATTC 6633) and *Aspergillus flavus* (MTCC 2798). All the bacterial strains were from the American Type Culture Collection purchased from Microbiologics (St. Cloud, MN, USA). They were maintained at -20 °C in 20% glycerol. *A. flavus* was obtained from MTCC Institute of Microbial Technology (Chandigarh, India). The bacterial cultures were activated by streaking on nutrient agar (NA) slant and incubating at 35 °C for 18-24 h. The fungal culture was grown on sabouraud dextrose agar (SDA) at 25 °C for 5 days. The bacterial cultures obtained on NA were dispensed in 0.85% saline and adjusted to a turbidity of MacFarland Standard No. 1, (3×10^8 cfu/ml). To obtain the inocula for disc diffusion assay, the suspension was diluted 100 times to give 3×10^6 cfu/ml. Density of the mould spore suspension in saline was 1,600/ml, which was determined by direct counting using a microscope (model BX51; Olympus, Tokyo, Japan) and counting chamber (Hellige Levy, Berlin, Germany).

Disc diffusion assay

Muller Hinton agar and SDA plates (HiMedia Laboratories, Mumbai, India) were prepared by pouring 15 ml sterile media to sterile petri dishes and allowing to solidify. 100 µl of prepared cell suspension (3×10^6 cfu/ml) was spread on plates using a sterile swab to get a uniform microbial growth and plates were allowed to air dry in a biosafety cabinet. Sterilised discs (Whatman No. 5 filter paper of

diameter 7 mm; Harsha Enterprises, Mumbai, India) were placed on the surface of the agar media and immediately impregnated with 30 µl (3.75 mg) of the prepared extract. Antibiotic discs of vancomycin and amoxycillin (6 mm) were used as reference positive control for bacterial cultures and cycloheximide was used as the control for the mould culture. 30 µl DMSO was used as vehicle control. Plates were kept at ambient temperature for 30 min to allow diffusion of extracts prior to incubation. The bacterial plates were incubated at 35 °C for 24 h. The fungus plates were incubated at 25 °C for 5 days. After the period of incubation, inhibition zones including diameter of the disc were measured. All tests were performed in triplicate and the bacterial activity was expressed as the mean of inhibition diameter (mm) produced.

Broth dilution assay for determination of minimum inhibitory concentration

The method outlined by Ericsson and Sherris (1971) was used for the assay. The Gram-positive bacteria used in the assay were *L. monocytogenes* (ATCC 13932), *S. aureus* (ATTC-12600), *B. cereus* (ATTC-11778) and *B. subtilis spizizenii* (ATTC 6633). The procedure involved two-fold dilutions of the dried pomace powder in 5 ml Muller Hinton broth dispensed in test tubes. The final concentration of the pomace powder in the Muller Hinton broth tubes was 1.25, 0.625, 0.3125 and 0.07625 mg/ml. The bacterial cultures obtained on NA were dispensed in 0.85% saline and adjusted to MacFarland Standard No.1 as was done for the disc diffusion assay. The bacterial suspension was diluted 10 times so that 0.1 ml contained approximately 10^6 cfu/ml. Following overnight incubation at 35 °C, the tubes were examined for visible bacterial growth as evidenced by turbidity. The lowest concentration of the extract that prevented growth represented the minimum inhibitory concentration (MIC).

Minimum bactericidal concentration

The minimum bactericidal concentration (MBC) was determined by plating out the broth culture from each test tube (of the MIC assay) into plate count agar plates which were then incubated at 37 °C for 24 h. The MBC is the concentration at which no growth is observed in the plates.

Preparation of wheat bread with/without dried carambola (*Averrhoa carambola*) pomace powder

Commercial wheat flour was procured from the local market in Mysore, India. It had 11.2% moisture, 0.51% ash, 10.5% gluten, 24.5 ml sedimentation value and 381 s falling number. Compressed yeast (Tower brand, Mumbai, India), sugar powder, vegetable fat (Hindustan Unilever Ltd., Bangalore, India) were procured from local market for the study. Breads were prepared in triplicates using straight dough method according to the standard 'Remix' baking test with slight modification (Irvine and McMullan, 1960). Fat at 1% level was included in the formulation. Dried carambola pomace powder was used in the formulation of bread by replacing wheat flour by 0, 5, 10 and 15% levels. The pomace powder was hydrated with water in the ratio of 1:3. Flour (100 g), fresh yeast (2.0 g), fat (1.0 g), sugar (2.5 g) and salt (1.0 g) were mixed in a planetary mixer for 4 min at 61 rpm. Dough was fermented for 90 min at 30 °C and 75% relative humidity with a knock back, moulded after 25 min, proved for 55 min and finally baked for 25 min at 220 °C. Breads prepared from wheat flour and with different levels of carambola pomace powder were cooled to room temperature, packed in polypropylene bags till further analyses.

Evaluation of breads

Breads were evaluated for its weight; and volume was measured using rapeseed displacement method (Mallock and Cook, 1930). Objective measurement of texture (crumb firmness) was carried out in a texture analyser (TAHDi; Stable Micro Systems, Godalming, UK) according to the AACC (2000) method. A bread slice of 25 mm thick was compressed by 25% using a 10 kg load cell and a plunger of 36 mm diameter at 100 mm/min crosshead speed.

Sensory evaluation of breads

Six panellists, who were briefed about the characteristics beforehand, carried out the sensory evaluation for crust and crumb characteristics on a 7-point hedonic scale by assigning different scores for various parameters. Samples were presented as whole bread for appearance, half-bread for crumb colour; texture by hand feel, and 2 cm slices for mouthfeel and eating quality. The data were subjected to statistical analysis using analysis of variance (ANOVA) followed by Duncan's multiple range test at significance level of $P<0.05$ (Steel and Torrie, 1980).

Determination of rope spore count in bread on storage

The rope spore count of the bread samples was determined by the method outlined in the 'Compendium of methods for the microbiological examination of foods' of the American Public Health Association (Washington, DC, USA; Stevenson and Segner, 2001). For each of three kinds of breads namely, (1) bread with no preservatives; (2) with chemical preservatives; and (3) with 5% carambola pomace but with no chemical preservatives were prepared as described earlier. Four sets of each kind were used in the assay. One set of each kind was analysed within 1 h of cooling after the baking process. The other three sets were kept for incubation at 35 °C, the optimum temperature for growth of *Bacillus* spp. Samples were drawn after 24, 48 and 72 h of incubation and analysed for rope spore count.

Related parameters that have a bearing on the survival and growth of bacteria, namely pH, titratable acidity and water activity were also determined at the time of rope spore count assay.

For determining rope spore count, 50 g of the bread crumb (the inner portion of the baked bread) was weighed and transferred to 450 ml of sterile 0.1% peptone and mixed well. 10, 1 and 0.1 ml volumes of the peptone water suspension were pipetted into separate 100 ml portions of melted dextrose tryptone agar contained in 250 ml flasks and held at 45 °C. The flasks and a control flask were submerged in a boiling water bath for 15 min. After heating, the flask contents were cooled to about 45 °C and contents of each flask were poured into 5 sterile plates in approximately equal volumes. When the agar had solidified, the plates were inverted and incubated at 35 °C for 48 h. The rope spore colonies are grey-white, vesicle like, becoming at first drier and finally wrinkled. The total colonies with these characteristics on the set of 5 plates from each flask were counted and rope spore count per gm determined.

Determination of pH, total acidity and water activity of the breads

pH determination of the loaves was measured by stomaching one slice of bread with distilled water and then the pH was recorded using Eutech Instruments, pH 510 (Singapore). For titratable acidity determination, 20 g sample of each loaf of bread was soaked in 100 ml of water, filtered and an aliquot was titrated against standard solution of sodium hydroxide (0.1 N). Acidity was expressed in terms of acetic acid per g sample (AOAC official method 947.05; AOAC, 1995). For water activity determination, a portion of the homogenised samples was determined using a Novasina water activity determining equipment (Lab master; Novasina AG, Lachen, Switzerland) according to the manufacturer's instructions.

3. Results and discussion

Physico-chemical characteristics of dried carambola fruit pomace

Carambola juice is known to be rich in oxalic acid (95.8 mg/g), which may result in the formation of oxalates in the human system; however, the pomace dried powder obtained after the expression of juice contains only 0.15 mg/g thus making it safe for use in food preparations. The total dietary fibre content of pomace was 71%, insoluble and soluble fibre being 61 and 10%, respectively (Table 1). Chau *et al.* (2004b) have also reported the high level of insoluble fibre-rich fractions including insoluble dietary fibre, alcohol insoluble solid and water-insoluble solid (46.0-58.2 g/100 g) of pomace. Physical properties of the dried pomace such as water holding capacity, oil holding capacity and bulk density were 2.5 g/g, 0.69 g/g and 0.41 g/cm^3, respectively.

Table 1. Physico-chemical properties of carambola (*Averrhoa carambola*) fruit pomace powder.[1]

Parameters	Quantities
pH	3.3±0.01
Titratable acidity	0.234±0.03
Oxalic acid (mg/g)	0.15±0.05
Starch (%)	6.3±0.2
Total sugars (mg/g)	59.2±0.12
Reducing sugars (mg/g)	54.0± 0.18
Insoluble dietary fibre (% on dry weight basis)	61.0±1.2
Soluble dietary fibre (% on dry weight basis)	10.0±0.9
Bulk density (g/cm^3)	0.41±0.02
Water holding capacity (g/g)	2.5±0.04
Oil holding capacity (g/g)	0.69±0.01

[1] Mean value of three replicates ± standard deviation (n=3).

In vitro antimicrobial activity of pomace

The antimicrobial susceptibility of bacterial cultures to the pomace was studied. The results obtained by the qualitative test of disc diffusion assay is presented in Table 2. It was observed that only the Gram-positive bacteria, namely the non-spore forming *L. monocytogenes* and *S. aureus*, and the spore formers namely, *B. cereus* and *B. subtilis*, were sensitive to the extract as evidenced by the formation of an inhibition zone. The Gram-negative bacteria did not show any inhibition of growth. No inhibition of growth was observed on the mould culture *A. flavus* used in the study. Greater sensitivity of Gram-positive bacteria than Gram-negative bacteria to herbal extracts has also been observed by Lee *et al.* (2002). The results of the studies on MIC of the susceptible bacteria are presented in Table 2. All the four Gram-positive bacteria examined were sensitive to a concentration of 1.25 mg/ml of the fibre extract. The MBC was also found to be the same as no growth was observed on further sub-culturing of the tubes into fresh solid medium. These results prompted to study the use of pomace in the preparation of bread for not only general quality improvement particularly in terms of fibre content but also for the control of rope spoilage by Gram-positive bacteria.

Quality characteristics of carambola pomace incorporated breads

The effect of the addition of different levels of carambola pomace powder to the wheat flour on the prepared breads is presented in Table 3. Increasing levels of the pomace powder from 0 to 15% significantly decreased the volume of the breads from 585 to 410 cm^3 ($P \leq 0.05$), thereby also decreasing the specific volume significantly ($P \leq 0.05$) from

Table 2. Susceptibility of microbial cultures to carambola (*Averrhoa carambola*) fruit pomace powder extract.[1]

Microorganism	Susceptibility index (inhibition zone in mm)
Escherichia coli serovar 01:K1:H7 (ATTC-11775)	no zone
Salmonella enterica subsp. *enterica* serovar Typhimurium (ATTC-25241)	no zone
Listeria monocytogenes (ATCC 13932)	13.7 (0.58)
Staphylococcus aureus (ATTC-12600)	11.67 (0.58)
Bacillus cereus (ATTC-11778)	13.67 (0.58)
Bacillus subtilis spizizenii (ATTC 6633)	12.00 (0)
Aspergillus flavus (MTCC 2798)	no zone

[1] The standard deviation is given in parenthesis (n=3); 3.75 mg in 30 µl of the extract contained in dimethyl-sulfoxide. The diameter of the paper disc is 7.0 mm.

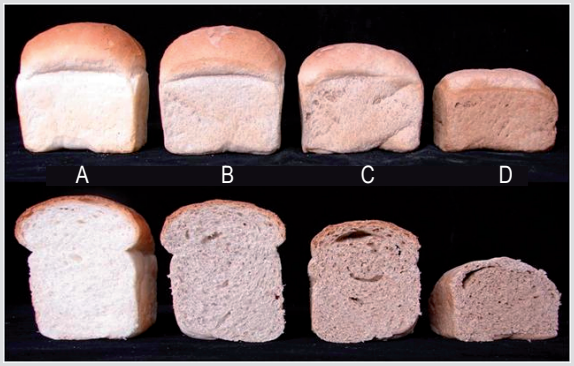

Figure 1. Photograph of breads with different levels of carambola (*Averrhoa carambola*) fruit pomace powder. (A) Control; (B) 5% pomace; (C) 10% pomace; (D) 15% pomace.

Table 3. Effect of carambola (*Averrhoa carambola*) fruit pomace powder on physical and sensory characteristics of breads.[1,2]

Carambola pomace (%)	Volume (cm^3)	Specific volume (cm^3/g)	Texture (g force)	Crust[3]		Crumb[3]			
				Shape	Symmetry	Colour	Grain	Texture	Overall quality
0	585±3.4a	4.25±0.11a	568±3.3d	6.0a	6.0a	6.0a	6.0a	6.0a	6.0a
5	575±2.8b	3.98±0.13b	649±4.2c	6.0a	5.5b	5.0b	5.5b	5.5b	5.5b
10	510±3.8c	3.35±0.09c	820±6.9b	5.5b	5.0c	4.5c	5.0c	5.0c	4.5c
15	405±2.3d	2.5±0.08d	1,041±8.3a	5.0b	4.5d	4.0d	4.0d	4.5d	3.5d
SEM	2.5	0.06	3.43	0.06	0.08	0.11	0.19	0.18	0.21
df	8	8	20	20	20	20	20	20	20

[1] Values are means ± standard deviation (specific volume: n=3; texture: n=6); values in a particular column followed by different letters differ significantly ($P<0.05$).
[2] SEM = standard error of mean; df = degrees of freedom.
[3] Crust and crumb characteristics were evaluated on a 7-point hedonic scale.

4.25 to 2.50 g/cm^3. Similar results have been reported when multigrain mix was used with the wheat flour (Indrani *et al.*, 2010). Şeker *et al.* (in press) reported that 5% gilaburu pomace incorporated cake had the highest overall acceptability score with increased amount of total phenolic content and radical-scavenging activity compared to control cake. According to the study, evaluation of gilaburu pomace waste as ingredient in developing alternative functional and/or value-added products by the food industry could be suggested.

The shape of the breads became flat at higher levels of pomace addition (Figure 1). The crumb grain of breads containing 0 and 5% pomace was medium fine and became dense and compact at 10 and 15% incorporation of pomace. This indicates that the retention capacity reduced with increasing levels of pomace. Similar results were reported by Chen *et al.* (1988), wherein breads were prepared from hard wheat flour by partially replacing with dry apple fibre and hydrated apple fibre (hydration 1:7 for 12 h). As the concentration of fibre increased, water absorption, mixing time, and bread weight increased, whereas the loaf volume decreased. On the other hand, the addition of hydrated apple fibre had a less deleterious effect on the bread making quality. The compression force required for control bread was 568 g which increased to 649 and 820 g on addition of 5 and 10% of pomace respectively and at 15% incorporation the compression force doubled. With regard to eating quality, breads with 5% pomace had fruit flavour and were acceptable. At 10 and 15% levels of

pomace, the breads possessed perceptible sour taste and the crumb colour became brownish, which made the breads unacceptable. Similarly, bread containing 5% apple pomace was acceptable according to (Masoodi and Chauhan, 1998).

Characteristics of bread on storage

The rope spore count was determined in the three kinds of baked bread stored at 35 °C and is presented in Table 4. It was observed that in all the three samples the count was less than 1 $\log_{10}$ count/g within 1 h of storage. The rope spore count ($\log_{10}$/g) of the bread sample with no preservatives (acetic acid and propionates) rose to 2.7, 5.62 and 8.76 $\log_{10}$ count/g after 24, 48 and 72 h, respectively. Besides, *B. subtilis*, the primary causative bacteria of rope spoilage, other strains of the genus *Bacillus* were also found to be present. As expected, the rope spore count in the bread with chemical preservatives showed less than 1 $\log_{10}$ count/g even after 72 h of storage. Experimental breads with no chemical preservatives but containing carambola pomace powder showed similar low count rope spores of 0.6 $\log_{10}$/g even after 72 h of storage. The percentage reduction was more than 99% (6 log reduction) in breads containing either chemical preservatives or carambola pomace. The antimicrobial effect was observed even though the experimental bread contained only 5% of the pomace powder.

It has been observed that the deterioration of bread texture is due to slime being formed as a result of the combined effect of the proteolytic and amylolytic enzymes produced by *Bacillus* strains (Viljoen and Von Holy, 1997). *B. subtilis* was identified as the prime cause of rope spoilage but recent molecular assays have now revealed other *Bacillus* species being the cause of ropy bread. Thus isolates of *Bacillus clasusii* and *Bacillus firmus* have also been identified (Pepe *et al.*, 2003) as causing ropy bread.

The pH of the bread with no preservatives (control) was found to be 5.98 at 0 h which reduced to 5.85 in 72 h (Table 4). The pH of the bread with synthetic preservatives remained between 5.59 and 5.69 during 72 h of storage while that of the experimental bread with carambola pomace powder ranged from 5.23 to 5.27 during the period of storage. The incorporation of the carambola pomace powder in the dough resulted in slight lowering of the pH. The decrease in pH may not be the reason for the inhibition of growth of *B. subtilis* in the experimental bread because the range of pH for growth for this genus is 5.0 to 9.0. Considering the D values of many *Bacillus* spp., it is not surprising that many survive the baking process during which the temperature in the core of the bread is 97 to 101 °C for a few minutes (Röcken and Voysey, 1995; Rosenkvist and Hansen, 1995). Leuschner *et al.* (1998) reported the D_{100} of *B. subtilis*, *Bacillus pumilis* and *B. licheniformis* to be 14, 10 and 56 min at 100 °C in brown soda bread of pH=7-9. The baked bread used in the present study had a pH of less than 6.0. Addition of the carambola pomace powder led to decrease in the pH of the bread. Rodríguez *et al.* (2010) observed that when the pH was reduced from 6.4 to 3.6 there was reduction in the thermal resistance of *Bacillus* spp. Palop *et al.* (1996) also observed that the thermal resistance of *B. licheniformis* at 99 °C was 20 times higher at pH=7 when compared to pH=4.

The titratable acidity of the experimental bread in which carambola pomace powder (Table 4) was incorporated was also higher (0.17) compared to that of the other two kinds of bread (0.12-0.13 average). This factor and that of pH, together with the chemical constituents of the carambola pomace powder added to the lowered thermal resistance of the *Bacillus* species during the baking process. There was not much difference in the water activity of the three samples at the three periods of storage and was in the range of 0.914 to 0.955 (Table 4).

The chemical preservatives used to increase shelf life of bread are sodium propionate and acetic acid. Pattison (2003) has reported that calcium propionate was not as effective as the sodium salt, in inhibiting the growth of *Bacillus* strains. Sodium propionate is effective against *B. subtilis* and against moulds but is ineffective against yeasts. Sorbic acid is effective in controlling mould growth in bakery products at level of 0.125 to 0.3%. Acetic acid and acetates are also effective against yeasts and bacteria but on a w/w basis they are less effective than propionates in controlling microbial spoilage. Above a certain concentration, acetic acid adversely affects the organoleptic quality of the baked products while propionic acid has been reported to cause irritability, restlessness, inattention and sleep disturbance in some children (Dengate and Ruben, 2002; Spicher, 1983) and may even cause cancer tumour (Rosenquist and Hansen, 1998).

These observations have led to research on controlling microbial spoilage in bread by the use of natural ingredients. The approach has been mainly in two directions. One is the use of sour dough and the other is the use of natural substances rich in known preservatives. Sour dough is used as an ingredient for acidification, leavening and production of flavour compounds and for bio preservation of bread (Clarke *et al.*, 2004; De Vuyst, 2007; Hansen, 2004; Katina *et al.*, 2005; Sadeghi, 2008). Many researchers have also reported the high resistance of sourdough breads to the microbiological spoilage by moulds and rope-forming bacilli (Hassan and Bullerman, 2008; Mentes *et al.*, 2007; Sadeghi, 2008; Ryan *et al.*, 2008; Valerio *et al.*, 2009). In fact, acidic sourdough is an ancient way of improving flavour, texture and microbiological shelf life of bread used in Mediterranean countries (Katina, 2005). Incorporation of raisin extract rich in propionates into the dough has been found to control microbial growth in bread (Wei *et al.*,

Table 4. Characteristics of baked breads during 72 h storage at 35 °C.

Parameter	Storage period (h)	Control bread with no preservatives	Control bread with synthetic preservatives	Bread with 5% carambola pomace
Rope spore count (log_{10}/g)	0	0.7	0.48	0.48
	24	2.7	0.7	0.602
	48	5.62	0.48	0.3
	72	8.76	1.0	0.602
pH	0	5.98	5.59	5.23
	24	6.03	5.69	5.20
	48	5.91	5.67	5.23
	72	5.85	5.69	5.27
Titratable acidity as acetic acid	0	0.13	0.16	0.18
	24	0.13	0.13	0.16
	48	0.11	0.11	0.16
	72	0.11	0.11	0.15
Water activity	0	0.914	0.941	0.934
	24	0.925	0.952	0.939
	48	0.923	0.951	0.948
	72	0.938	0.955	0.949
Dietary fibre (g/100 g)	–	2.2	2.15	6.75

2009). Following increased demand for foods with natural ingredients in Britain, many bakeries resorted to the use of vinegar rather than propionates as preservatives leading to increased incidences of rope (Von Holy and Allan, 1990). The dietary fibre content in the bread without preservatives and bread containing synthetic preservatives was around 2.2 g/100 g bread. By addition of 5% carambola pomace the dietary fibre increased to 6.75 g/100 g, i.e. by 3 times compared to the bread without preservatives (Table 4). This is due to high amount of dietary fibre in carambola pomace as presented in Table 1.

There is thus a trend worldwide as mentioned earlier to do away with chemical preservatives in the process of baking bread. What was of great concern in this study was the appearance of the bread with no preservatives, even though it had a high rope spore count of 8.76 log_{10} count/g. It appeared normal with no offensive odour (Figure 2) particularly when compared with the bread with added preservatives and with the carambola pomace incorporated bread. Many *Bacillus* species including the rope spore formers have been found to be the cause of food borne disease. According to Gilbert (1981) and Kramer and Gilbert (1989), high numbers of *B. subtilis* and *B. licheniformis* in foods may cause a mild form of food borne illness and consumption of ropy bread has been associated with food borne illness in reports from Canada (Todd, 1982) and the UK (Sockett, 1991). The documentation of cases of food poisoning due to consumption of ropy bread involves nausea, vomiting, diarrhea, headache and chills (Kirschner and Von Holy, 1989; Voysey, 1989).

Figure 2. Photograph of bread crumb at the end of storage period of 72 h at 35 °C. (A) Control bread with no preservatives; (B) control bread with synthetic preservatives; (C) bread with 5% carambola (*Averrhoa carambola*) pomace.

Foodborne illness caused by *Bacillus* spp. is under-reported, as symptoms are generally mild and self-limiting. The levels of *B. cereus* required to produce toxin is approximately 10^5 spores/g of food, while higher spore levels (10^6-10^9 spores/g) are required for *B. licheniformis* and *B. subtilis* (Katina, 2005; Lund, 1990).

4. Conclusions

In the present study an approach has been made to incorporate a natural substance, namely carambola fruit pomace, which is a fruit waste that has antimicrobial properties. Incorporation of the pomace also increased the dietary fibre content by 3 times in the bread. The incorporation of pomace has thus enriched the nutritional quality of the product without disturbing the sensory appeal. It was also able to inactivate the *Bacillus* species that were the potential rope spore causing bacteria in bread.

The observations are an important step in ensuring public health through the use of a natural substance to control potential food borne pathogens in bread instead of the addition of synthetic preservatives.

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