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ORIGINAL ARTICLE

Effect of storage on Spirulina platensis powder supplemented breads

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Abstract The aim of the present study was to develop breads by incorporating Spirulina platensis powder and to study their organoleptic acceptability, antioxidant activity, storage conditions and shelf life of value added acceptable breads. Spirulina platensis powder was mixed with wheat flour at different levels (2, 4, 6 and 8%) to prepare nutritive breads. Breads were developed and organoleptic evaluations were performed to find out the most appropriate level of supplementation of Spirulina platensis powder. The results showed that 6% Spirulina supplemented breads provided higher contents of total phenolic content (1.79 mg GAE/g) and antioxidant activity (16.51%). On the basis of organoleptic characteristics, overall acceptability scores indicate that Spirulina incorporated breads can be stored up to 2 days at room temperature and up to 4 days at refrigeration temperature without any preservatives. Fat acidity content and total bacterial count were found increased as time period increased but did not exceed the acceptable limit as given by BIS (Bureau of Indian Standards). Spirulina supplements are available in the market but normally people does not consume it may be because of less awareness or other reasons but if this super food is incorporated in food products like bread, which is consumed by everyone will aid to food security and malnutrition of developing countries.

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Introduction

Since past years many countries are facing nutritional problems like protein energy malnutrition and other macro and micronutrient deficiencies; iron, vitamin A, vitamin D and B-complex deficiencies being the important one (Anupama 2000; Matondo et al. 2016). Indian women, more than (50%) are at high risks of anemia, which results in maternal deaths, low birth weight and low learning ability in children (Nguyen et al. 2018). Moreover, consumers prefer refined food or junk foods which are handy, easy to cook in less time or convenience foods. It may be because of urbanization, industrialization, participation of women in working force and it's a menace to a society (Singla et al. 2012; Bolanho et al. 2014). In view of health, common food products which are available in the markets like bread must be nutritious. In many countries people eat breads as their staple food, flour is the important ingredient in making bread which form grid and help other ingredients in mix to form the dough. Wheat is the only cereal which has protein (gluten), which determines the strength, shape and structure of the dough. Different varieties of breads are available in the market, wheat, refined flour, multi grain etc. which consists of abundant in fat, carbohydrates and energy, whereas lacking in micronutrients (Navacchi et al. 2012). Therefore, nutritional supplementation of food products which contain more amounts of refined flour and their consumption is more can be a boon to use as carrier of nutrients due to their simple manufacturing process, better shelf life, high acceptability and consumption. One of such

foods which can deal with multi nutrients and has remarkable name is "Spirulina".

Spirulina is a latin word meaning 'helix' or 'spiral', denoting the physical configuration of the organism when it forms swirling, microscopic strands. Spirulina, which is commercially known as Arthospira, is one of the blue-green algae because of the presence of chlorophyll, carotenoid and phycocyanin pigments. Spirulina is a simple one celled form of algae that thrives in high temperature, high alkaline fresh-water in sunlight (Navacchi et al. 2012). Spirulina can be available with the common name for human and animal food supplements produced primarily from two species of Cyanobacteria i.e. Arthrospira platensis and Arthrospira maxima. Cultivation of Spirulina platensis is mostly seen in Asian countries i.e. India, Japan and China whereas, Spirulina maxima is mainly found in Central America. Spirulina from Chad lake in Africa and Texco lake in Mexico have been harvested as a source of food (Guldas and Irkin 2010; Dineshkumar et al. 2016).

Nutrient content of Spirulina are (50–70%) protein, (20%) carbohydrate, (5%) lipid, (7%) minerals and (3 to 6%) moisture. Therefore, Spirulina platensis powder has high amount of protein with low lipid, low-calorie and cholesterol-free which can be compared with meat and meat products in respect to protein. Many therapeutic properties are present in Spirulina platensis such as hypocholesterolemic, immunological, antiviral and antiglutagenic effects, decrease blood pressure, lipids and glucose in blood, weight reduction, increase in the population of microorganisms in the intestinal flora, improvement in immunologic response, renal protection against heavy metals and medicines (Mc Carty 2007; Bolanho et al. 2014). Spirulina contain high amount of good quality protein with all essential amino acids in perfect balance and also derived of high concentration of minerals and B-complex vitamins specially thiamine which is usually found in animal tissues (Guldas and Irkin 2010; Dineshkumar et al. 2016). Markets are filled with Spirulina supplements in form of powder, flakes, capsule and tablet. It is full of nutrients with so many health benefits but still its use is restricted in food products. Therefore, utilization of nutrient-rich Spirulina platensis powder in from of supplemented food products may be encouraged to improve the nutritional status of the general population, especially in developing countries.

Materials and methods

Procurement of raw material

Procurement of Spirulina platensis powder was done from Herbo Nutra Wholesale Trader, New Delhi. WH-1105, variety of wheat was procured from Wheat and Barley Section of Department of Genetics and Plant Breeding, CCSHAU, Hisar and other ingredients were availed from local market.

Preparation of bread

Flours were divided into the ratio Wheat flour: Spirulina (98:2, 96:4 and 94:6) and were sieved. Dough was formed by adding 3 gm yeast, 4 gm oil, 1.75 gm salt and ± 60 ml water. First proofing was done for 30 min; scaling and round up of dough. Second proofing was done for 20 min again scaling and round up of dough. Final proofing was for 60 min in greased tin moulds and baked them for 5-8 min at 240 °C. Finally cooling of breads for 30 min at room temperature.

Antioxidant activity

Total phenolic contents

Total phenolic contents were determined by the method of Singleton and Rossi (1965).

Reagents

(i) Folin-Ciocalteu reagent (1 N)
(ii) Sodium carbonate (Na_2CO_3): Dissolved 200 g anhydrous sodium carbonate in 800 ml water and brought to a boil. After cooling, added a few crystals of sodium carbonate and let it sit for 24 h at room temperature. Filtered through Whatman No. 1 filter paper and added water to 1 L.
(iii) Standard gallic acid

Preparation of calibration curve using gallic acid as standard Ten mg of standard gallic acid was accurately weighed and dissolved in 100 ml distilled water in a volumetric flask (100 µg/ml of stock solution). From the above stock solution, 0.1 to 1 ml aliquots were pipetted out into 25 ml volumetric flasks. Ten ml of distilled water and 1.5 ml of Folin Ciocalteu reagent were added and diluted according to the label specification to each of the above volumetric flasks. After 5 min., 4 ml of 20% sodium carbonate solution was added and volume was made up to 25 ml with distilled water. Absorbance was recorded after

30 min. at 765 nm and a calibration curve of absorbance vs concentration was plotted.

Procedure One g of sample was added to 15 ml of methanol (50%) and extracted for three times by maceration of 2 h. Then it was filtered and volume was made to 50 ml in volumetric flask with methanol (50%). One ml aliquot of the sample was taken in a test tube and diluted with 10 ml of distilled water. Then, 1.5 ml Folin Ciocalteu's reagent was added and allowed to incubate at room temperature for 5 min, then 4 ml of 20% (w/v) Na_2CO_3 was added, adjusted with distilled water up to the mark of 25 ml, agitated and left to stand for 30 min at room temperature Absorbance of the sample was measured at 765 nm. Quantification was done on the basis of a standard curve of gallic acid. Results were expressed as µg gallic acid equivalents (GAE) and percentage w/w.

2, 2-Diphenyl-1-picrylhydrazyl (DPPH)

The DPPH RSA of sample extracts was evaluated by the DPPH method of Hatano et al. (1988).

Reagents DPPH: 2.5 mg/l in methanol

Procedure An aliquot of (0.1 ml) methanolic solution containing 20-100 µg of crude phenolic extract of sample was mixed with 2 ml of methanol and then added to a methanolic solution of DPPH (1 mmol/l, 0.25 ml). The mixture was vortexed for 10 s, left to stand at room temperature for 30 min and then its absorbance was recorded at 517 nm against methanol blank. A control was measured using the same procedure except that methanol was used instead of extracts (at zero min). The percent of DPPH radical discoloration of the sample was calculated according to the equation (%) discoloration:

$$\text{DPPH RSA } (\%) = A_{control} - A_{sample} \times 100 / A_{control}$$

where, ($A_{control}$) = absorbance for the control, (A_{sample})- = absorbance for the sample

Shelf life

Sensory evaluation

Sensory evaluation of stored breads at the ratio 2, 4 and 6 per cent (room and refrigerator temperature) were periodically evaluated by a panel of ten semi trained judges for colour, appearance, aroma, texture, taste and overall acceptability using a 9 point Hedonic Rating Scale. All ten panel members filled the consent form to analyzed breads sincerely.

Fat acidity

The fat acidity was determined by the standard method of analysis (AOAC 2000).

Reagents

i. Benzene-alcohol-phenolpthalein solution (0.02%): To one litre benzene, one litre alcohol and 0.4 g phenolphthalein was added and mixed.
ii. Potassium hydroxide solution (0.0178 N).

Procedure Ten gram sample was extracted with petroleum ether on Soxhelet apparatus. The solvent of the extract was completely evaporated on steam bath. The residue was dissolved in extraction flask with 50 ml benzene-alcohol-phenolpthalein solution and titrated with standard potassium hydroxide (1 g/lt) to orange pink colour. Blank titration was made on 50 ml benzene-alcohol-phenolpthalein and this value was substracted from titration value of the sample. Fat acidity was calculated as mg of potassium hydroxide required to neutralize free fatty acids of 100 g of flour.

$$\text{Fat acidity } = 10 \times (T - B)$$

where, T = ml of KOH required to titrate sample extract, B = ml KOH required to titrate blank

Microbial count

Microbial count was determined in the stored products by using the method of (Harrigan and Margaret 1996).

Composition of plate count agar media

Agar - 4.5 g
Tryptone - 2.5 g
Yeast extract - 1.25 g
Dextrose - 0.5 g
PH - 7.0 ± 0.2 at 25 °C
Distilled water - 500 ml

Sterilization of media and glassware Plate count agar media was prepared in distil water and autoclaved at 121.6 °C (15psi) for 25 min. The media was then cooled up to 45 °C and used for conducting the test. All the glassware was sterilized in hot air oven at 160 °C for 2 h.

Procedure One g of sample was dissolved into 9.0 ml of sterilized distiled water blank and shaken thoroughly. One ml of 10^{-1} dilution was taken and dissolved into another

9.0 ml sterilized water blank. This was 10^{-2} dilution. Similarly other dilutions were made. 0.1 ml of 10^{-3}, 10^{-4} and 10^{-5} dilutions were poured in petri plate containing PCA media. After solidification, the plates were kept in inverted position and plates were incubated at 30 ± 2 °C for 24-48 h. Number of colonies were counted and colony forming unit (cfu) was calculated by using formula:-

No. of colonies × dilution factor × volume taken = cfu /g of sample

Statistical analysis

The data obtained were analyzed by (ANOVA) (Sheoran and Pannu 1999).

Results and discussions

Results of antioxidant activities of control and Spirulina supplemented breads are given in (Table 1). Whole wheat flour bread contained (11.80%) of antioxidant activity. Whereas, Spirulina fortified breads had significantly ($P \leq 0.05$) higher amount of antioxidant activity as compared to control bread. At the level of Six per cent Spirulina powder supplemented bread was observed to have significantly higher antioxidant activity of 16.51 per cent which were followed by 4 per cent (15.10%) and 2 per cent (13.37%) Spirulina supplemented breads.

Results of sensory evaluation of control and Spirulina supplemented breads are given in (Tables 2, 3). Overall acceptability scores of whole wheat flour bread up to 2nd day were found in the range of 7.77 to 8.00, which were 'liked moderately' by the panel members. Whereas, overall acceptability scores of Spirulina powder incorporated breads were 7.60 to 8.10 in 2 per cent Spirulina supplemented bread, 7.48 to 7.60 in 4 per cent incorporated bread and 6.60 to 7.16 in 6 per cent incorporated bread, respectively up to second days of storage. However, overall acceptability scores of fortified breads after second day were decreased as the period of storage was increased at room temperature and fell in the category of 'neither liked nor disliked' to 'disliked slightly' by the panel members. Hence, breads can be stored at room temperature up to second day without any food preservatives.

Table 1 Mean and standard deviation of total phenolic content and antioxidant activity in *Spirulina* powder incorporated breads (dry matter basis)

Breads	Total phenolic contents (mg GAE/g)	DPPH (%)
Control (100% WWF)	0.62 ± 0.03	11.80 ± 0.74
Supplementation level (%) WWF: SP		
98: 2	0.85 ± 0.04	13.37 ± 0.99
96: 4	1.19 ± 0.06	15.10 ± 1.00
94: 6	1.79 ± 0.13	16.51 ± 0.85
CD ($P \leq 0.05$)	0.13	0.99

WWF whole wheat flour, *SP Spirulina* powder

Overall acceptability scores of whole wheat flour bread on 0, 2nd and 4th days of storage were 8.00, 7.70 and 6.60, respectively, which were 'liked moderately' to 'liked slightly' by the panalists. Whereas, overall acceptability scores of Spirulina incorporated breads were found to be in the range of 8.10 to 6.40, 7.60 to 6.30 and 7.16 to 6.20, respectively from day 0 to 4th day of storage period and these were 'liked very much' to 'liked slightly' by the panel members. On 7th day of storage, overall acceptability scores of whole wheat flour and Spirulina supplemented breads were found in the category of 'neither liked nor disliked'.

Fat acidity content of whole wheat flour and supplemented breads at room temperature were significantly ($P \leq 0.05$) increased up to 7 days of storage period (Table 4).

Whole wheat flour bread had 19.77 mg KOH/100 g on 0 day. Whereas, 2, 4 and 6 per cent Spirulina platensis powder supplemented breads had 19.35, 19.29 and 19.10 mg KOH/100 g on 0 day, respectively, which were found to be increased with increase in storage period i.e. 24.34, 32.00 and 51.10 mg KOH/100 g in 2 per cent, 24.00, 31.73 and 50.73 mg KOH/100 g in 4 per cent and 23.78, 31.25 and 50.00 mg KOH/100 g in 6 per cent Spirulina incorporated breads, respectively on 2nd, 4th and 7th days of storage, respectively at room temperature. Fat acidity in breads increased as the storage period increase, it might be due to the formation of free fatty acid by the hydrolysis of triglycerides.

The data on fat acidity content of wheat flour and Spirulina platensis incorporated breads at refrigeration temperature is presented in (Table 4). Fat acidity content of wheat flour and Spirulina platensis incorporated breads were also increased up to seventh days of storage period. Wheat flour bread had 19.77 mg KOH/100 g on 0 day. Whereas, 19.35, 19.29 and 19.10 mg KOH/100 g were found in 2, 4 and 6 per cent Spirulina platensis incorporated breads, respectively on 0 day. As the storage period increased, fat acidity levels in breads were also found increased. The fat acidity values were 21.46, 24.23 and 30.79 mg KOH/100 g in 2 per cent Spirulina platensis supplemented bread, 21.13, 23.51 and 30.32 mg KOH/100 g in 4 per cent Spirulina platensis fortified bread and 20.63, 23.00 and 30.14 mg KOH/100 g in 6 per cent Spirulina platensis supplemented bread, respectively on 2nd, 4th and 7th day of storage at refrigeration temperature.

Table 2 Mean and standard deviation of effect on organoleptic characteristics in breads incorporated with Spirulina powder (stored at room temperature)

Sensory Evaluation	Storage period (days)	Control	Supplementation level (%)			
			2	4	6	Mean
Overall acceptability	0	8.00 ± 0.16	8.10 ± 0.19	7.60 ± 0.18	7.16 ± 0.17	7.71
	2	7.77 ± 0.17	7.60 ± 0.21	7.48 ± 0.32	6.60 ± 0.26	7.36
	4	5.46 ± 0.20	5.15 ± 0.18	5.78 ± 0.17	5.20 ± 0.15	5.39
	7	5.30 ± 0.16	5.10 ± 0.13	4.50 ± 0.16	4.20 ± 0.15	4.77
Mean		6.63	6.48	6.34	5.79	
CD ($P \leq 0.05$)	Storage period = 0.26		Supplementation level = 0.10, Interaction = 0.1			

Table 3 Mean and standard deviation of effect on organoleptic characteristics in breads incorporated with Spirulina powder (stored at refrigeration temperature)

Organoleptic characteristic	Storage period (days)	Control	Supplementation level (%)			
			2	4	6	Mean
Overall acceptability	0	8.00 ± 0.16	8.10 ± 0.19	7.60 ± 0.18	7.16 ± 0.17	7.71
	2	7.70 ± 0.20	7.60 ± 0.36	7.50 ± 0.22	7.00 ± 0.10	7.45
	4	6.60 ± 0.33	6.40 ± 0.22	6.30 ± 0.22	6.20 ± 0.21	6.37
	7	5.30 ± 0.22	5.60 ± 0.21	5.50 ± 0.30	5.10 ± 0.46	5.37
Mean		6.90	6.92	6.72	6.36	
CD ($P \leq 0.05$)	Storage period = 0.27		Supplementation level = 0.27, Interaction = NS			

NS non-significant

Table 4 Mean and standard deviation of effect of storage on fat acidity (mg KOH/100 g) in breads incorporated with Spirulina powder (dry matter basis)

Types of breads	Storage period (days)						
	Room temperature				Refrigeration temperature		
	0	2	4	7	2	4	7
Control (100%WWF)	19.77 ± 0.58	24.42 ± 0.87	32.25 ± 1.20	51.56 ± 0.38	21.89 ± 3.34	24.59 ± 8.80	31.17 ± 14.73
Supplementation level (%) WWF: SP							
98: 2	19.35 ± 0.84	24.34 ± 0.12	32.00 ± 0.91	51.10 ± 0.59	21.46 ± 2.96	24.23 ± 8.82	30.79 ± 14.91
96: 4	19.29 ± 0.54	24.00 ± 0.34	31.73 ± 0.55	50.73 ± 0.50	21.13 ± 2.74	23.51 ± 8.69	30.32 ± 15.09
94: 6	19.10 ± 1.13	23.78 ± 0.73	31.25 ± 0.25	50.00 ± 0.60	20.63 ± 2.49	23.00 ± 7.92	30.14 ± 15.21
CD ($P \leq 0.05$)	Storage period = 4.02				Storage period = 3.01		
	Supplementation = 0.19				Supplementation = 0.20		
	Interaction = 3.02				Interaction = 2.00		

WWF whole wheat flour, *SP* Spirulina powder, *NS* non-significant

In case of supplemented breads, fat acidity content varied from 31.25 to 32.00 mg KOH/100 g at room temperature up to fourth days of storage and 30.14 to 30.79 mg KOH/100 g at refrigeration temperature up to seventh day of storage. Fat acidity values in breads were found within the permissible limit.

Total bacterial counts of control and Spirulina platensis incorporated breads samples stored at room temperature are described in (Table 5). Freshly whole wheat flour bread

Table 5 Viable counts of bacteria in breads incorporated with *Spirulina* powder stored at room and refrigeration temperature on different storage periods

Breads	Storage days Total bacterial count (cfu/g)						
	Room temperature				Refrigeration temperature		
	0	2	4	7	2	4	7
Control (100% WWF)	3.1×10^1	4.2×10^1	6.2×10^2	7.6×10^3	3.6×10^1	5.0×10^1	6.1×10^2
Supplementation level (%) WWF: SP							
98: 2	2.8×10^1	3.7×10^1	5.6×10^2	7.2×10^3	3.3×10^1	4.6×10^1	5.8×10^2
96: 4	2.6×10^1	3.5×10^1	5.1×10^2	6.9×10^3	2.9×10^1	4.4×10^1	5.6×10^2
94: 6	2.4×10^1	3.2×10^1	4.9×10^2	6.9×10^3	2.9×10^1	3.9×10^1	5.4×10^2

WWF whole wheat flour, *cfu* colony forming unit

prepared in hygienic condition, its bacterial count was observed 3.1×10^1 cfu/g, stored at room temperature, which increased as the storage interval increased. Highest total bacterial count 7.6×10^3 was recorded on seventh day which followed by 6.2×10^2 on forth day and 4.2×10^1 on second day of storage period. Almost similar trend was also observed in Spirulina platensis powder incorporated breads. On zero day, freshly prepared Spirulina platensis incorporated breads at levels (2, 4 and 6%), their total bacterial counts were obtained as 2.8×10^1, 2.6×10^1 and 2.4×10^1 cfu/g in samples, respectively. The number in counts increased as storage period increased. Among the Spirulina platensis incorporated breads, highest bacterial counts were obtained from the breads made from 2 per cent Spirulina platensis supplemented in whole wheat flour whereas, the lowest counts were obtained in 6 per cent Spirulina platensis supplemented bread on 2nd, 4th and 7th days of storage interval.

On the other hand, total bacterial counts of whole wheat flour bread and Spirulina platensis incorporated bread samples stored at refrigeration temperature (Table 5) were recorded lower as compared to the bread samples stored at room temperature.

On 2nd, 4th and 7th days of storage total bacterial count in whole wheat flour bread was observes as 3.6×10^1, 5.0×10^1 and 6.1×10^2 cfu/g, respectively. Likewise increasing trends were also found in 2, 4 and 6 per cent Spirulina platensis incorporated breads. The range of bacterial counts were found from 2.9×10^1 to 3.3×10^1 cfu/g on second day, 3.9×10^1 to 4.6×10^1 cfu/g on fourth day and 5.4×10^2 to 5.8×10^2 cfu/g of sample on seventh day of storage period. Highest bacterial counts were observed in 2 per cent Spirulina platensis incorporated bread while lowest bacterial counts were obtained in 6 per cent Spirulina platensis incorporated breads. Bacterial counts were found within acceptable limits as stated by BIS that bacterial count must not exceed 10,000 cfu/g in cereal based products.

Conclusion

Spirulina platensis incorporated (2, 4 and 6% levels) breads were found acceptable by the panel members and found nutritionally higher as compared to their respective control breads. Among the supplemented breads, 6 per cent Spirulina platensis powder supplemented breads exhibited higher amount of all the nutritional parameters. Hence, Spirulina platensis incorporated breads may be recommended because of its health beneficial properties like antiviral, immunological, hypocholesterolemic and antiglutagenic effects. Further intervention study can also be done with pre and post evaluation effects.

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