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Solid State Fermentation of Bee-Collected Pollen

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A B S T R A C T

This research work aimed at obtaining a novel natural food product from pollen, safe and improved nutritional value, to be used as a dietary supplement or a functional ingredient for formulating other foods. Bee-collected pollen subjected to lactic acid fermentation using lactic acid bacteria *Lactobacillus lactis* and its effect on some of the natural characteristics of the pollen were studied. The optimum conditions for the pollen fermentation were provided that are, anaerobic condition for solid state fermentation at 35^0 C for first 96 hours, then 20^0 C for next 72 hours, and optimum moisture content was 35-40%. The process was characterized byt the production of lactic acid and decrease in pH and sugar content. As a result of this project the proteins were increased by 1.53%, total sugars were decreased by 32.6 %, lactic acid content increased by 1.35%, total free amino acid content increased by 1.99%, total poly-phenol content decreased by 1.8%, increment in all minerals and radical scavenging activity increased by 18.86% fermented pollen. The solid state fermentation of the bee pollen by Lactic acid bacteria were effective to increase its nutritional value.

Introduction

Pollen exists for a time as an independent unit and thus contains most nutrients, essential for life. Man has long been the consumer of pollen and pollen containing food. In many living organisms like insects, pollen is essential for their life cycle, being rich particularly in protein (Wakhle, 1981). Once bee pollen was defined in legislation as food, the nutritional value of this product became important. It contents high concentration of reducing sugars, essential amino acids, fatty acids, minerals and abundant in proteins and vitamins (Campos *et al.*, 2010).

The bee pollen is used as a nutritional source for human from ancient times, but the pollen wall structure resists the digestion and decay, so they remain intact in digestive tracks of the animals and the pollen contents will not be released in the digestive tract (T'aiand Cane, 2000). So it is suggested that to increase the digestibility and palatability of pollen it should be treated with chemicals or acids to loosen the pollen wall.

It is well known that honey bees do not consume pollen directly. They store it in combs by adding their saliva containing lactic acid bacteria. The lactic acid bacteria have

found in the honey stomach (Olofsson*et al.*, 2011). This pollen then undergoes the lactic acid fermentation. The lactic acid fermentation increases shelf life, improve palatability, digestibility and nutritional value (Gilliam, 1997).

Materials and Methods

Sample collection

Pollen collected from *Apismellifera* bee hives located in the area of Jalgoan Village of Pune district of Maharashtra. (N 18 0 23.275' and E 075 0 02.592') where the hive was used for pollination of Sunflower (*Helianthus annuus*) crop. Pollen was collected using a pollen trap by attaching to the entrance of the hive. The culture of lactic acid bacteria *Lactobacillus lactis* was collected from Vidyapratisthan School of Biotechnology-Culture Collection laboratory, Baramati.

Solid state fermentation

Pure culture of lactic acid bacteria *Lactobacillus lactis*, 20ml were inoculated in 100g of bee-collected pollen in a sterile glass jar, under aseptic condition. It was incubated at 35^0 C temperature for the first 96 hours, then 20^0 C for next 72 hours under anaerobic conditions. After the fermentation process, the product was stored at 4^0 C to avoid nutrient losses and spoilage. The process of fermentation was monitored by estimating an increase in lactic acid content and a decrease in pH.

Determination of moisture content in pollen

The moisture content of pollen was determined by the method described in AOAC, (1997). The 1.5g pollen sample heated in a hot air oven at 130^0 C until the constant weight of pollen obtained.

Estimation of total acidity in pollen

Total acidity was estimated by acid-base titration with 0.1 N Sodium hydroxide and phenolphthalein as an indicator (AOAC, 1990). The acidity was determined by the following formula

$$\text{Percent of Lactic acid} = \frac{\text{ml of NaOH used x Conc. of NaOH x 0.090}}{\text{Weight of sample}}$$

Where 0.090 is an equivalent weight of Lactic acid.

Estimation of protein content in pollen

Protein content was estimated by Folin-Lowry method (Lowry*et al.*, 1951) the 0.2 ml sample were allowed to react with 2ml alkaline copper sulfate solution followed by 10 min incubation at 37^0 C then added 0.2ml of Folin-Ciocalteau phenol reagent for 10 min at 37^0 C and the absorbance was read at 660nm using Spectrophotometer (JASCO 630). Bovine serum albumin was taken as a standard protein. The value of total protein expressed as mg/g of pollen.

Total sugar content estimation in pollen

The total sugar content was estimated by the Anthrone method (Hedge *et al.*,1962) with some modifications. The 100mg pollen sample washydrolyzed in 5ml 2.5N HCl in boiling water bath for 3 hours followed by cooling neutralized with solid sodium carbonate and volume make up to 100ml. the 1ml prepared sample was allowed to react with 2 ml anthrone reagent prepared in 75% H_2SO_4. The absorbance was taken at 630nm using Spectrophotometer (JASCO 630). And from the standard curve, the concentration of sugar was estimated. Glucose was taken as a standard.

Quantitative determination of total free amino acids

Free amino acid extraction and quantitative estimation of pollen sample performed by the method given by Sadasivam and Manickam (1992). 500mg of pollen sample mixed with 10ml 80%ethanol and homogenized. Then centrifuge and collect the supernatant. Repeat method twice with residue. Evaporate ethanol by using a boiling water bath and dissolve the residue in distilled water. Take 2 ml of this sample and add 1 ml Acetone- Ninhydrin reagent (0.1% Ninhydrin in acetone) then incubate it in boiling water bath for 20 min followed by cooling under running tap water. And take absorbance at 570nm. Express the free amino acid content in pollen protein in terms of mg of glycine equivalent per gram of pollen.

Estimation of total poly-phenol content in pollen

The 1g pollen sample homogenized and extracted using methanol: water (1:1 v/v) as an extraction solvent. The solvent was evaporated at 60^0 C for 4 hours. Dissolve extract in sterile distilled water and store in the refrigerator. The 0.5ml of a prepared sample taken and add 2.5ml of 1N Folin-Ciocalteau reagent followed by 0.5ml 4% Na_2CO_3. Take gallic acid as a standard and absorbance were read at 750nm using Spectrophotometer (JASCO 530).

Determination of radical scavenging activity of pollen

For ABTS [2,2'- azinobis (3-ethyle-benzothiazoline-6- sulfonic acid)] assay, the procedure followed the method of Arnao*et al.*,(2001). The stock solutions included 7mM ABTS solution and 2.4 mM potassium persulphate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 14 hours at room temperature in the dark. The solution was then diluted by mixing 2 mL ABTS solution with 50 mL methanol. The ethanolic pollen extract used for determining the antioxidant activity were prepared by extracting 1 g of crushed pollen in 15ml of 70% ethanol in a water bath at 70^0 C for 30 min. the next sample was centrifuged and the supernatant was stored at 4^0 C in the refrigerator. The ethanolic pollen extract 1ml were allowed to react with 2ml of the ABTS solution and the absorbance was then taken at 734nm after 5 min. incubation.

The ABTS scavenging capacity of the compound was calculated as,

$$\text{ABTS radical scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control x 100}}$$

Where Absorbance of control is the absorbance of ABTS radical in methanol; Absorbance of sample is the absorbance of an ABTS radical solution mixed with the sample.

Determination of mineral content of the pollen

The minerals Ca, Cu, Mn, Mg, Fe, Zn were determined after the incarnation of 1 g pollen at 555ºC in Muffle furnace, until a constant weight was obtained. Next, the ash was solubilized in 25ml of HNO_3 50%, heated in a water bath for 30 min, filtered and the minerals determined by Atomic Absorption Spectrophotometer (AAS) (Parkin-Elmar Analyst 200).

Results and Discussion

In the fermentation process the lactic acid produced by the microbial actions. The lactic acid bacterium utilizes the carbohydrates present in the pollen and produces the lactic

acid. The lactic acid content after 168 hrs fermentation was 6.10% in fermented pollen respectively while as in control set the acid content remains constant. The lactic acid content slightly increased after fermentation of the bee-collected pollen. The Gilliam (1997) has been reported 115 fold increase in titratable acidity in stored pollen load by bees. The increase in acidity indicates active fermentation process. Carlos*et al.*,(2012) reported high acid production up-to 400 meq/kg in the fermentation of bee pollen by different probiotic starters he also reported eventually decrease in the sugar content of the pollen. In pollen fermentation process the total sugar content has found decreased eventually. In fermented pollen, it was decreased by 31.60% within 168 hrs of the fermentation process.

The pollen contains various sugars like glucose, fructose and minute amount of sucrose. But after fermentation the sugars concentration get changes (Table 1). Stanciu *et al.,* (2011) reported 52.16% fructose in bee pollen and 57.51% in bee bread that is naturally fermented pollen by bee stomach bacteria. The glucose was 42.59% in bee pollen and 35.88% bee bread, the sucrose accounted for 1.57% and 0.12% in bee pollen and bee bread respectively. The total sugar content in pollen was reported 40 % by Campos *et al.,* (2008). Because of the increase in the acidity, the pH of the bee pollen was decreased. As per the reports of De-Grandi-Hoffman *et al.,* (2013) naturally the pH of the pollen is 5.5-6.5. It varies by botanical source. The pH of the bee bread ranges between 3.5 and 4.5. The fermentation found successful to decrease pH up to 4.2 in fermented pollen. These pH ranges are effective to avoid the growth of nonessential microorganisms in pollen. So it will effective to increase the shelf life of the final product. It will also improve palatability, digestibility and nutritional value (Gilliam, 1997) (Fig. 1).

The water content in the dried pollen was reported 6-8% by Campos*et al.*(2008). In the fresh bee-collected pollen ranges from 20-30 % (Campos *et al.,* 2010). The high water content required for the microbial action in pollen for fermentation. The moisture content of the pollen will vary plant to plant, region by region and seasonal variations also found in bee pollen, it will range from 14-30%, but for fermentation the amount of water is necessary is above 36% for microbial activity (Carlos *et al.,* 2012). For solid state fermentation, the fresh pollen was collected in the cold season that having water 34%.

As per the evaluation, the protein content of the Sunflower pollen collected by the bees was 26.8 ±0.2 mg/g and after fermentation, the protein content found increased by 12.53 ±0.2 mg/g. Campos *et al.*, (2010) reported the protein content of the bee pollen ranged 10 to 40% and Stanciu *et al.,* (2011) reported it ranges 15 to 28% and in bee bread, it was 16.94 to 30.23%.

The total free amino acid content of the *Helianthus annuus* pollen was estimated that was 80.76 ±0.20 mg/g. In fermented pollen, it was found increased. In fermented pollen, it was 99.95 ±0.13 mg/g. As per Bhunia and Mondal (2012) free amino acid content in different pollen ranges between 53.5 - 68.5 mg/g. As per DeGrandi-Hoffman *et al.,* (2013) mention, the free amino acid also can be incorporated into proteins. The amino acid content of fermented pollen or bee bread probably depends not only on the botanical source of the pollen but also on the bees and microflora added by them (Table 3).

The polyphenol content of the bee pollen found decreased after fermentation. It was also reported by Carpes *et al.,* (2009). In fermented pollen, it decreased by 18mg/g. Polyphenols are responsible for radical scavenging activity or anti-oxidant activity. The free radical scavenging activity in bee

pollen has been found 67.27%, but it will increase after fermentation up to 86.06%. The free radical scavenging activity was found effective in the treatment of diseases like Diabetes, cancer, hypertension (Pandey and Rizvi, 2009).

Table.1 Lactic acid and total sugar content during the fermentation process

Time in Hours	Lactic acid %		Total Sugar %	
	Control	Fermented Pollen	Control	Fermented Pollen
0	4.12	4.2	41.83	42
24	4.18	4.23	41.23	30.8
48	4.3	4.86	40.2	28
72	4.32	5.49	39.6	29.6
96	4.35	6.09	39	14.8
120	4.35	6.09	38.7	12.6
144	4.36	6.09	38.4	10
168	4.36	6.1	38.5	10.4

*Values in the table are Arithmetic mean of three replications

Table.2 Change in nutritive value after fermentation

Pollen	Moisture content %	Protein (mg/g)	Total sugar content %	Total free amino acid content (mg/g)	Poly-phenol content (mg/g)	Radical scavenging activity (%)
Control	34.66	26.8	43	80.76	33.33	67.27
Fermented - pollen	46.66	41.33	10.4	99.95	28.66	86.06

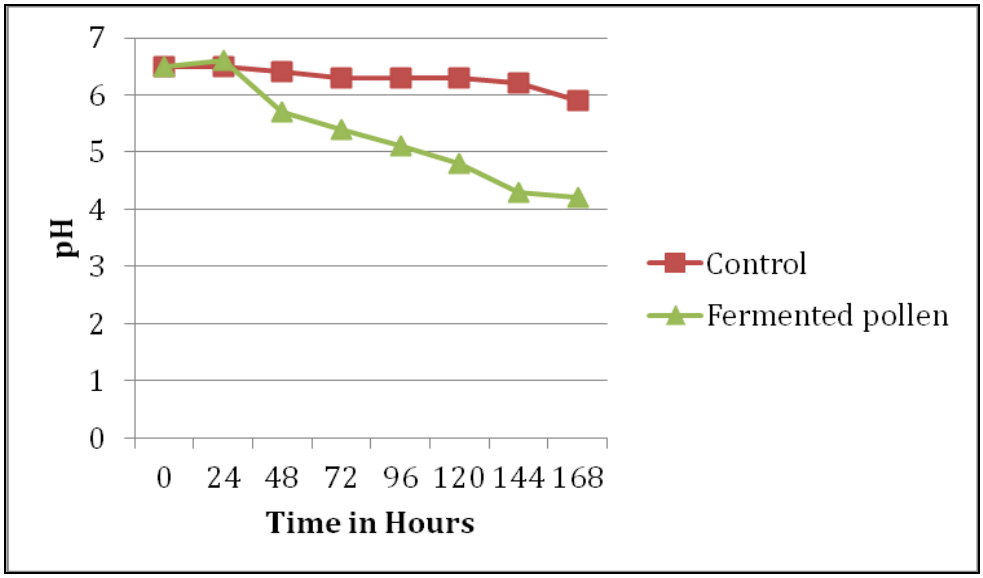
*Values in the table are Arithmetic mean of three replications

Table.3 Change in mineral content after fermentation of pollen

Minerals in Pollen (mg/g)						
Pollen type	**Ca**	**Fe**	**Mg**	**Mn**	**Cu**	**Zn**
Control	0.154	0.116	7.032	0.017	0.003	0.171
Fermented pollen	0.362	0.96	9.403	0.205	0.071	1.032

*Values in the table are Arithmetic mean of three replications

Figure.1 Change in pH during the fermentation process



The pollen also contains the essential minerals the predominant minerals of the pollen are Phosphorous followed by potassium, calcium, and magnesium (Carpes*et al.*, 2009). In sunflower pollen, the amount of the minerals found to be dominant are magnesium followed by calcium, iron, and zinc. The lower amount of manganese and copper. The phosphorous and potassium are not tested in the pollen samples. The amount of all minerals found increased slightly in fermented pollen then the control (Table 3).

In conclusion, the solid-state fermentation of the bee collected pollen was performed successfully at the 35^0C temperature for 4 days followed by 20^0C for next 4 days. The fermentation process was characterized by estimating lactic acid production, decrease in sugar content and pH. As per the results and observation, it is suggested that the pure culture of the lactic acid bacteria, *Lactobacillus lactis* was found an effective starter for the solid-state fermentation of the bee collected pollen to increase its nutritive value.

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