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RESEARCH ARTICLE

Optimized production of gamma poly glutamic acid (γ-PGA) using locally isolated *Bacillus megaterium* bacteria for potential application in some food products

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Abstract

Poly-γ-glutamic acid (γ-PGA) is a water-soluble amino acid biopolymer produced by bacterial fermentation. γ-PGA functioned as a precursor of protein development, including glutamate, an amino acid that provides an umami taste, especially in foods rich in proteins. Thus, in this study, the gamma polyglutamic acid (γ-PGA) obtained from genetically improved local isolate *Bacillus megaterium* was added to a few food products and the quality changes of the foods were evaluated. Crude γ-PGA samples were produced from genetically modified locally isolated *Bacillus megaterium* biosynthesis. The bacteria culture medium (g/L) consists of 100 g glucose, 20 g ammonium nitrate, 2.5 g corn soaking liquid, 0.5 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$ and 0.005 g $MnCl_2.2H_2O$ and is added with 3 % v/v of the bacteria culture. Then, sensory evaluation was conducted on three types of food products (mayonnaise, mushroom soup and chicken sausage) after γ-PGA was added in different concentrations. The addition of γ-PGA was different for each food product based on the food standard concentrations: mayonnaise, 0.4 %, 0.7 % and 1 % respectively; chicken sausages, 0.2 %, 0.5 % and 0.8 % respectively and mushroom soup, 0.2 %, 0.25 % and 0.3 % respectively. Potato starch was tested on food products, respectively, as a comparison with a commercial thickener agent. The results showed that the sensory evaluation reported no significant differences ($p > 0.05$) with the samples that contained 2.5 % potato starch. The (γ-PGA) synthesized from genetically modified, locally isolated *Bacillus megaterium* improved the tested food products' texture, taste and palatability.

Keywords: alternative thickener; *Bacillus megaterium*; flavor enhancer; food ingredients; gamma polyglutamic acid (γ-PGA)

Introduction

Poly-γ-glutamic acid (γ-PGA) is an anionic biopolymer polypeptide, wherein D- and L-glutamate units are shaped via γ-amide linkages in the concentration of 30 % and 70 % for D- and L-glutamate respectively (1). Due to its structural characteristics, γ-PGA has several promising functions in the food industry. γ-PGA is water soluble and has high water holding capacity and viscosity, respectively. PGA is classed into two isoforms: poly-α-glutamic acid (α-PGA) and poly-γ-glutamic acid (γ-PGA). α -PGA is produced chemically and γ-PGA is synthesized via bacterial fermentation. The chemical structure in which the peptide bonds are between the amino group of glutamic acid and the carboxyl group at the end of the glutamic acid side chain determines it as γ-PGA (2, 3). The γ-PGA is produced via ribosome unbiased manner, in which D- and L-glutamates are copolymerized in a single filament (γ -DL-PGA) with the aid of the membrane γ-PGA synthase (4, 5). It is unfastened from protease assault as its miles comprise D-and L-glutamic acid units linked through α-amino and γ-carboxylic acid (5, 6).

Microbial manufacturing of γ-PGA is complicated and the polymer can be produced by recombinant technology (7). γ-PGA has been produced on a large scale by using bacteria, especially from *Bacillus subtilis* species. Both can be composed of L-glutamic acid residues (γ -L-PGA), D-glutamic acid residues (c-D-PGA), or each L- and D-glutamic acid residue (γ -LD-PGA). γ -PGA is different compared to other proteins since the glutamate is polymerized inside the cell by γ -amide linkages and in a ribosome-impartial manner (8). Therefore, substances that inhibit the translation of proteins, consisting as chloramphenicol, have no impact on the manufacturing of γ -PGA. Due to the γ -linkage of its issue glutamate residues, γ -PGA is resistant to proteases, which cleave α -amino linkages. Besides, γ -PGA is also known as an enzyme inhibitor (9-12). Microbial synthesis of γ-PGA is carried out by four following steps, racemization, polymerization, regulation and degradation. The above steps have a first-rate position within the PGA manufacturing with specific enzymes that are coded via respective genetic materials inclusive of, racE/glr, yrpC, pgsB, C, A, E, ComP-ComA regulator device, DegS-DegU, DegQ, SwrA systems, ywtD, dep or pgdS and ggt (13-15). γ -PGA can exist either within the water-insoluble free acid form or as its salt with numerous cations (Na, Mg_2, K, NH_4 or Ca_2), to act as a masking taste agent.

Therefore, it has been detected for various applications such as biodegradable plastics, flocculants, biological adhesives and food additives (16-19).

One of the certainly taking place resources of γ -PGA is the mucilage of natto (fermented soybeans), a traditional food in Japan. Natto mucilage containing γ -PGA substantially enhanced calcium solubility *in vitro* and *in vivo* in rats as well as the calcium content material in their bones (2, 20, 21). Some previous studies investigated the effect of γ -PGA on oil absorption and moisture loss respectively, in deep-fried doughnuts were also investigated (22, 23). Results have shown that at a frying time of four minutes and γ -PGA oil retention of 100 g (one hundred g dough)-1, was decreased fivefold in doughnuts containing γ -PGA [0.2 g (g dough)-1] compared to everyday doughnuts [0.7 g (g dough)-1] (22, 23). Hence, γ -PGA doughnuts had a better look and taste than regular doughnuts γ -PGA is therefore a potential oil-reducing agent in deep-fats fried meals. The addition of γ -PGA to wheat bread reduced its hardness via storage. γ -PGA also will be more advantageous to the rheological and thermal properties of wheat dough. The addition of γ -PGA proved to enhance the texture of sponge cake (24).

Thus, this study aims to utilize gamma polyglutamic acid (γ-PGA) produced by genetically modified local isolated *Bacillus megaterium* in chicken sausage, mayonnaise and mushroom soup to evaluate its softness, thickness and palatability. Potato starch was also added, respectively, into the food products to compare the sensory evaluation outcomes. Besides, there is limited literature reporting the usage of gamma polyglutamic acid (γ-PGA) synthesized by genetically modified local isolated *Bacillus megaterium.*

Materials and Methods

Biosynthesis of γ-PGA from genetically modified local isolated *Bacillus megaterium*

Bacteria strain

Isolated and genetically improved *Bacillus megaterium* bacteria were obtained from the laboratories of the Biotechnology Department, College of Applied and Biotechnology, Al-Nahrain University, Iraq for this study. The strain was modified by utilizing protoplast fusion and ultraviolet mutagenesis methods in 250 mL conical flask fermentation scale.

Inoculation medium

The medium was prepared according to the method (25), including (g/L); (40 g) glucose, (10 g) yeast extract, (0.5 g) $MgSO_4$, (1 g) K_2HPO_4, (1 g) KH_2PO_4, (2.5 g) NaCl, (0.1 g) $MnSO_4.H_2O$.

Bacteria culture medium

The medium consists of glucose (100 g), 20 g ammonium nitrate 2.5 g corn soaking liquid, 0.5 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$ and 0.005 g $MnCl_2.2H_2O$ respectively.

Parameters of fermentation condition

The microbial fermentation of the production medium was carried out under environmental conditions that included a temperature of 34 °C, initial medium pH of 6.5, rotation speed of 180 rpm and an incubation period of 72 hr.

Identification of gamma poly glutamic acid (γ-PGA)

Detection of gamma poly glutamic acid (γ-PGA) from the bacteria culture medium

The qualitative detection and quantitative determination of gamma poly glutamic acid (γ-PGA) produced in the production medium was carried out by applying the Thin Layer Chromatography (TLC) technique described (26). The quantification of (γ-PGA) produced by *B. megaterium*, the mobile phase for (TLC) was prepared undergone using the separation solution method. A mixture of solvents; Butanol (20 mL): acetic acid (20 mL): and distilled water (20:20:80 mL) were added into the culture medium and homogenously shaken for a minute. The stationary phase for TLC was a silica gel 60F 254 precoated thin-layer chromatography (TLC) plates (20 cm × 20 cm, 0.20 mm) from Macherey-Nagel (Germany) that were used for amino acids analysis. The solutions (samples of γ-PGA) were applied to the points marked with a pencil on a line at equal distances where the points are within one centimetre of the edge of the plate. All stains were dried with a dryer before placing the plate in the glass chamber. Then, a ninhydrin detector (0.2 % ethyl alcohol) was added to detect the color of the separated γ-PGA. The separated γ-PGA was extracted and dried before being tested. The dried sample was placed in test tubes containing 5 mL ethanol (75 %) and the absorbance was read at the wavelength of 570 nm. The concentration of γ-PGA was calculated by referring to the standard curve of commercial γ-PGA shown in (Fig. 1) (R^2=0.9958).

Extraction, purification and crystallization of gamma poly glutamic acid (γ-PGA)

The γ-PGA was purified by the method reported (27, 28). A volume of 1 L culture solution was collected from the fermentation process of genetically modified locally isolated *Bacillus*

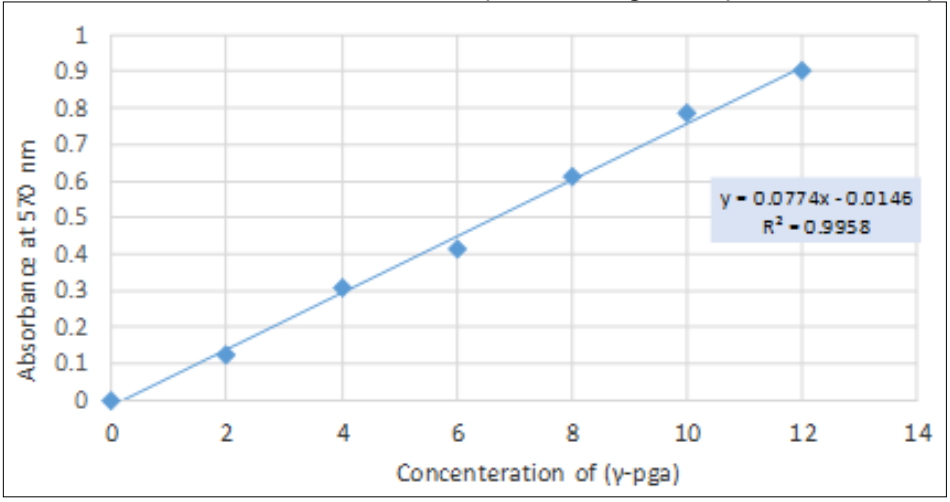

Fig. 1. Concentration of γ-PGA.

megaterium. The centrifugation process was carried out at a speed of 5000 rpm for 20 min and then the supernatant containing γ-PGA was poured into four volumes of methanol. The mixture was kept at 4 °C for 12 hr. Crude γ-PGA was collected by centrifugation of 50 mL volume at 10,000 rpm and 4 °C for 30 min. Next, the precipitations were dissolved in 50 mL distilled water and any insoluble impurity was removed by centrifugation. Then the medium was acidified by titrating it with 0.1 N of hydrochloric acid (HCl) until pH 3.2. Next, the solution was kept in the refrigerator (4 °C) for 48 hr until crystals of γ-PGA were formed and precipitated in the beaker. The crystals were collected by filtration using a Buchner funnel, then washed with distilled water for twice in succession to purify them from impurities, if any. The dry process was carried out in an electric air oven at 60 °C to turn γ-PGA into dry material.

Manufacture of chicken sausages

In preparing chicken breast sausages, with the addition of γ-PGA to those mixtures as the treatment, the formula was according to the following components: 75 % chicken breast, 15 % abdominal fat, 8.5 % boiled potato dough and 1.2 % salt with the addition of γ-PGA at three concentrations: 0.2 %, 0.5 % and 0.8 % respectively to chicken sausage mixture. Meanwhile, potato starch was incorporated with chicken sausage mixture in three concentrations: 1 %, 2 % and 3 % to chicken sausage mixture respectively separated from the samples with γ-PGA addition. Chicken sausage mixture without additives was used as a control (29).

Manufacture of mayonnaise

The method was used to manufacture mayonnaise samples (Table 1). The product was divided into 7 parts with equal weights and γ-PGA from the fermentation process was added into three concentrations; 0.4 %, 0.7 % and 1 % respectively compared to three different concentrations of potato starch; 1 %, 2 % and 3 % respectively with a control (Mayonnaise without any additives). Each sample is symbolized by a letter (A, B, C, D, etc.) (30).

Manufacture of mushroom soup

The method of preparing mushroom soup powder was according to the ingredients in Table 2.

The thickening material γ-PGA from the fermentation process was added with three concentrations; 0.10 %, 0.25 % and 0.3 % respectively and potato starch was used as a commercial thickening material for comparison with three concentrations: 1 %, 2 % and 3 % respectively, for each sample. The control was mushroom soup without any additives. Each sample is symbolized by a letter (A, B, C, D, etc.) (31).

Sensory evaluation of food products

Sensory evaluation of food products incorporated with gamma-poly glutamic acid (γ-PGA)

The effect of adding γ-PGA for improving the properties of chicken sausages, mayonnaise and mushroom soup in enhancing the taste, distinctive texture and sensory evaluation of manufactured samples was evaluated.

Sensory evaluation of appearance and gustatory

Appearance sensory evaluation scores were determined for the characteristics of general shape, apparent color and texture. The gustatory sensory evaluation scores for manufactured sausages were determined for characteristics including flavor, tenderness and general acceptability as shown in Table 3. An amount of 30 participants from the College of Agriculture, University of Baghdad participated in those evaluations (32).

Sensory evaluation of mushroom soup

Sensory evaluation was conducted on mushroom soup samples containing three concentrations of γ-PGA under study and three concentrations of potato starch with a control sample (without addition). The form of sensory evaluation was based on (33).

Statistical analysis

The statistical program SAS-Statistical Analysis System was used for the analysis process. The data is used to evaluate the effect of adding different concentrations of γ-PGA and potato starch on sensory evaluation characteristics of chicken sausage, mayonnaise and mushroom soup products. The significant differences between the means were compared using the least significant difference (LSD) test (34).

Results and Discussion

The amount of γ-PGA produced from the synthesized process was 7 g/L. This extracted γ-PGA was added to the food products at the concentrations mentioned in the prior sections. A sensory evaluation study was performed to evaluate the food product properties.

Table 1. Ingredients used in manufacturing mayonnaise samples

Ingredients	Vegetable oil	Whole eggs	Vinegar	Mustard flour	Mustard	Salt	Sugar
Weight (g)	66.66	26.16	2.02	0.33	2.33	1.20	0.80

Table 2. Ingredients used in manufacturing mushroom soup samples

Ingredients	Mushroom powder	Corn flour	Milk powder	Salt	Cumin powder	Black pepper	Sugar
Weights (g)	40	15	135	25	5	5	25

Table 3. Appearance and taste sensory evaluation scores for chicken breast sausages

Code	General appearance	Virtual color	Flavor	Tenderness	Overall acceptance
9	Excellent	Excellent	Excellent	Excellent	Very acceptable
8	Very good	Very good	Very good	Very good	Acceptable
7	Good	Good	Good	Good	Average
6	Acceptable	Acceptable	Medium	Medium	Slightly acceptable
5	Acceptable to average	Acceptable to average	Acceptable	Low	Fairly acceptable
4	Fairly acceptable	Fairly acceptable	Fairly acceptable	Little	Somewhat unacceptable
3	Somewhat unacceptable	Somewhat unacceptable	Slightly non-existent	Medium	Moderately rejected
2	Unacceptable	Unacceptable	Non-existent	High hardness	Rejected
1	Totally unacceptable	Completely rejected	Totally non-existent	Very high hardness	Completely rejected

Sensory evaluation was conducted on mayonnaise samples containing three concentrations of γ-PGA under study and three concentrations of potato starch with a control sample (without addition).

The form of sensory evaluation for the mayonnaise product was based on (35).

Sensory evaluation of mushroom soup

Sensory evaluation was conducted on mushroom soup samples containing three concentrations of γ-PGA under study and three concentrations of potato starch with a control sample (without addition). The form of sensory evaluation was based on (33).

More desirable than control (R): slightly = 6, average=7, much=8, very much=9

Less desirable than the control (R): a little = 4, average=3, much=2, very much=1

Sensory evaluation of food products (Chicken sausages, mayonnaise and mushroom soup)

Sensory evaluation is a valuable tool in evaluating food acceptance, which is useful for improving the product and maintaining its quality as well as is important in the development of new products (36). γ-PGA was included in this experiment as a thickening material in the manufacture of chicken breast sausages and the effect of its addition at different concentrations on the degrees of sensory and gustatory evaluation. The product was also compared with other samples which included the addition of different concentrations of potato starch and the control treatment was a sample without any addition of thickener. The results in (Table 4) showed that there was no significant difference ($P > 0.05$) in the general appearance, color and flavor for all samples. Whilst there were significant differences ($P > 0.05$) for the freshness or tenderness characteristic between the different treatments with the control sample. The results of the sensory evaluation of the tenderness characteristic and the general acceptability characteristic of treatments B and E showed that there was no significant difference when adding γ-PGA at 0.5 % and the potato starch tenderness at 2%. The average scores of the sensory evaluation of the characteristics of tenderness were around 8.95 and 8.98 respectively and for overall acceptance, it was 8.72 and 8.87 respectively. (Please insert Table 4 here)

The results corresponded with previous study whereby the researcher was adding γ-PGA as a substance that enhances the texture of sausages. The sausage was manufactured by adding some materials-free meat such as powdered milk, soybean proteins and starch. Consumers preferred the product fortified with γ-PGA more than other types of sausages that excluded γ-PGA from their ingredients (37). The foods prepared with the addition of γ-PGA as a tenderness substance require an addition percentage within the range of 0.1 - 0.9 % (22). Therefore, the percentage of adding 0.5 % γ-PGA to the chicken sausage product under study was within the acceptance range. An approximation to the texture characteristic of the added treatment including 2 % potato starch was in the recommended percentage by the manufacturers.

Table 4. The effect of adding γ-PGA on the sensory evaluation of food products (Chicken sausages, mayonnaise and mushroom soup)

Chicken breast sausages

Additive material (%)	Addition ratios (%)	*Code samples	Appearance characteristics		Taste characteristics		
			General appearance	Virtual color	Flavor	Tenderness	Overall acceptance
Control sample	0	A	7.60 ^{a}	8.54 ^{a}	7.36 ^{a}	5.60 ^{c}	6.60 ^{c}
(γ-PGA)	0.2	B	7.48 ^{a}	8.57 ^{a}	7.40 ^{a}	6.63 ^{bc}	7.3 ^{bc}
	0.5	C	7.36 ^{a}	8.42 ^{a}	7.26 ^{a}	8.95 ^{a}	8.72 ^{a}
	0.8	D	7.38 ^{a}	8.59 ^{a}	7.40 ^{a}	7.54 ^{ab}	8.1 ^{ab}
	1	E	7.41 ^{a}	8.67 ^{a}	7.46 ^{a}	6.80 ^{bc}	7.5 ^{bc}
potato starch	2	F	7.44 ^{a}	8.70 ^{a}	7.24 ^{a}	8.98 ^{a}	8.87 ^{a}
	3	G	7.62 ^{a}	8.72 ^{a}	7.46 ^{a}	7.1 ^{bc}	7.9 ^{ab}
LSD value	------	-------	NS	NS	NS	1.79*	1.062*

Mayonnaise

Additive material (%)	Addition ratios (%)	*Code samples	Attributes				
			Color	Odor	Flavor	Consist of	Overall acceptance
Control sample	0	A	5.00 ^{a}	5.00 ^{a}	5.00 ^{a}	5.00 ^{d}	5.00 ^{d}
	0.4	B	5.11 ^{a}	5.00 ^{a}	5.05 ^{a}	6.36 ^{cd}	6.02 ^{cd}
(γ-PGA)	0.7	C	5.16 ^{a}	5.05 ^{a}	5.10 ^{a}	8.26 ^{a}	8.57 ^{a}
	1.0	D	5.00 ^{a}	5.20 ^{a}	5.20 ^{a}	7.56 ^{ab}	7.72 ^{ab}
	1	E	5.00 ^{a}	5.20 ^{a}	5.00 ^{a}	7.26 ^{ab}	6.77 ^{bc}
potato starch	2	F	5.26 ^{a}	5.10 ^{a}	5.20 ^{a}	8.32 ^{a}	8.72 ^{a}
	3	G	5.26 ^{a}	5.20 ^{a}	5.20 ^{a}	6.72 ^{bc}	6.67 ^{bc}
LSD value	------	-------	NS	NS	NS	1.47*	1.511*

Mushroom soup

Additive material (%)	Addition ratios (%)	Attributes					
		Taste	Color	Flavor	Appearance	Solubility and miscibility	Overall acceptance
Control sample	0	9.40 ^{a}	8.53 ^{a}	9.20 ^{a}	7.20 ^{b}	7.00 ^{b}	41.32 ^{b}
	0.2	9.45 ^{a}	8.49 ^{a}	9.40 ^{a}	7.70 ^{b}	8.05 ^{ab}	43.23 ^{ab}
(γ-PGA)	0.25	9.55 ^{a}	8.48 ^{a}	9.25 ^{a}	9.15 ^{a}	8.75 ^{a}	45.17 ^{a}
	0.3	9.60 ^{a}	8.43 ^{a}	9.20 ^{a}	8.80 ^{a}	8.45 ^{a}	44.45 ^{ab}
potato	1	9.50 ^{a}	8.58 ^{a}	9.25 ^{a}	7.88 ^{ab}	8.25 ^{a}	43.55 ^{ab}
	2	9.54 ^{a}	8.63 ^{a}	9.33 ^{a}	9.25 ^{a}	9.00 ^{a}	45.62 ^{a}
starch	3	9.65 ^{a}	8.78 ^{a}	9.45 ^{a}	7.85 ^{ab}	8.50 ^{a}	44.11 ^{ab}
LSD value	------	NS	NS	NS	1.166*	1.188*	3.027*

Table 4 also depicts the sensory characteristics results of mayonnaise samples treated with different concentrations of γ-PGA compared to potato starch samples and the control treatment (without additives). The results showed that the color and flavor were not significantly affected when using different types and proportions of thickness additives. While there was a significant difference (P<0.05) for the thickness characteristics, the average sensory evaluation score increased to 8.26 when using γ-PGA at a concentration of 0.7 %. The result indicated 8.32 % consist of when using 2 % potato starch, which indicates the closeness of these two treatments in a much more desirable way compared to the control sample (R). Research indicates that γ-PGA has the potential to be an alternative thickener as the quality is similar to the commercial thickener. Besides, γ-PGA synthesis is a low-cost process and feasible production (38).

The results of the softness or consist of attribute were reflected in the average scores of the overall acceptance attribute, which indicated significant differences between all treatments except for the two treatments C and F, which were 8.57 % and 8.72 % respectively. Thus, the γ-PGA was added at the concentration of 0.5 % was the optimal concentration of the mayonnaise thickness and softness as well as desired by the panelists, compared to a concentration of 2 % of the potato starch samples.

To develop a dry soup, it must have the required quality, which is represented by the flavor, aroma, thickness and softness of the components included in the composition. It is recommended that the product must be free of unacceptable flavors and tastes, unacceptable odors and defects in texture and consistency (36). Table 4 shows the effect of adding different types and ratios of thickener substances on the results of sensory evaluation for some attributes of mushroom soup.

The results showed that adding thickeners in different concentrations and types did not significantly affect (P> 0.05) the color, flavor and taste of the soup ingredients. Adding the thickeners had a significant effect (P< 0.05) on the appearance, solubility and miscibility of the soup ingredients, which gave the best average of sensory evaluation at 9.15 % and 8.75 % respectively when the γ-PGA was added at a concentration of 0.25 %. It corresponded to the best concentration of potato starch at a concentration of 2 %. Mushroom soup mixed with γ-PGA improves the properties compared with using potato starch (39). The results of the statistical analysis did not show significant differences between the manufactured γ-PGA mushroom soup in a concentration of 88 % and the γ-PGA chicken soup, which obtained a concentration of 90 %.

Conclusion

In conclusion, the results show that γ-PGA synthesized from genetically modified local isolated *Bacillus megaterium* enhanced the texture, taste, of, thickener and palatability in the concentrations 0.7 %, 0.25 % and 0.5 % for mayonnaise, mushroom soup and chicken sausage respectively. Future studies will determine the potential application of the γ-PGA synthesized from genetically modified locally isolated *Bacillus megaterium* in various food products to improve its texture, thickness and softness as well as its nutritional value.

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Authors' contributions

EJAA carried out the bacteria culture medium preparation, participated in the biosynthesis process of genetically modified local isolated *Bacillus megaterium* and participated in the sequence alignment and drafted the manuscript. SKH carried out and participated in the biosynthesis process of genetically modified local isolated *Bacillus megaterium*. LKH participated in the design of the study and performed the sensory evaluation of food products after their manufacturing, statistical analysis and participated in the sequence alignment and drafted the manuscript. HK conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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