Lactic acid bacteria: economic propagation, chitinases activity, and enhancing viability by gel encapsulation

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Background
In a previous study, a lactic acid bacterium, *Enterococcus faecium*, was locally isolated from Egyptian soil and its ability to inhibit the growth of a test phytopathogen was proven.
Objective
The study was performed to assess the ability of the tested strain to grow on different media. The produced antifungal agent was investigated. Finally, the strain was encapsulated within different biopolymers to increase its viability.
Materials and methods
Several byproducts were tested and compared with the standard De Man-Rogosa-Sharpe medium. The antifungal activity was tested using the poisoned food technique. Chromatographic analysis of the fermentation medium was performed using high-performance liquid chromatography. Production of chitinase was confirmed by cultivating the test strain on chitin and estimating the amount of reducing sugars using the Somogyi method. The *E. faecium* cells were also encapsulated within soy protein isolate-alginate beads, gellan gum discs, and carboxymethyl cellulose beads.
Results and conclusion
The strain was able to grow on all of the tested byproducts and exerted a potent antifungal activity against *Fusarium solani*, especially when a very economic medium, mainly composed of whey, was used. High-performance liquid chromatography results confirmed the production of a number of organic acids that contributed in the inhibition of the fungal growth. The study also proved the production of chitinase enzymes, which apparently altered the chitinous layer present in the cell wall of *F. solani,* causing disintegration of the fungal cells. It was also shown that encapsulation of *E. faecium* increased its viability in soil as compared with the free uncapsulated strain.

Keywords:
alginate, byproducts, chitinase enzyme, lactic acid bacterium, soy protein-isolate

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Introduction

The use of lactic acid bacteria (LAB) for biocontrol, especially in agriculture, is a very promising approach to reduce not only the losses in agricultural crops but also the extensive use of hazardous chemicals and toxic pesticides [1]. This perspective mainly refers to the ability of this group of bacteria to produce a number of metabolites, including organic acids, antibiotics, diacetyls, and others [2,3]. These compounds were proven to have potent activity against both phytopathogenic fungi and bacteria [4–6]. Furthermore, LAB and their products are not only safe to be consumed but also have various health benefits [7].

Although LAB were, over the last decades, cultivated in the synthetic De Man-Rogosa-Sharpe (MRS) medium of De Man, Rogosa, and Sharpe [8], the use of more economic medium components such as whey, molasses, or other byproducts would be more appropriate. These substances, which are either unintentionally produced as byproducts of some industries or even rejected as wastes, were proven to contain several beneficial compounds that could be used as alternative to the more expensive medium components. For instance, whey, which is a liquid formed as a byproduct during the process of cheese production, was found to be composed of 93% of water and 50% of total solids from milk with lactose as a major component [9]. Moreover, sugarcane molasses, which is a byproduct of the sugar industry, were proved

to contain about 30–36% sucrose, 10–17% of both fructose and glucose, as well as some smaller quantities of polysaccharides [10].

As previously reported, the major cause of agricultural crop spoilage are fungi that propagate through spores, which have the ability to adapt to extreme and harsh growing conditions and to resist common biocontrol agents. However, chitin is a polysaccharide that represents a characteristic and essential component of the cell walls of all fungal species, which adds rigidity and structural support to the thin cells of the fungus [11]. Accordingly, the degradation of this layer results in the loss of many vital functions of the fungal cell wall, including its permeability, thus leading to a drastic, irreparable damage to the fungal cells.

On the contrary, many studies have proven that the fungal cell wall effectively degrades by different hydrolyze enzymes including chitinase [12]. Chitinase enzymes are a synchronized group of enzymes together forming an enzyme system that has the ability to degrade chitin into more simple sugars that could be easily consumed by different microorganisms. Therefore, the ability of any bacterial strain to produce chitinase enzymes permits its growth on chitin and could consequently allow it to alter the growth of certain fungi.

Unfortunately, although many studies currently focus on the use of LAB for the biological control of fungi, only a scarce number of researcher studies have studied the production of chitinase enzyme by LAB, and the reported results were consequently very limited. For instance, Leisner *et al*. [13], reported chitinase activity by only two out of 115 LAB strain tested belonging to different species, including Carnobacterium, Lactobacillus, Leuconostoc, and Weissella. Moreover, Beck *et al*. [14], identified the presence of chitin-binding protein, among other cell surface proteins of the probiotic bacterium *Lactobacillus plantarum* 299 v, without confirming the ability of this strain to produce chitinase. More recently, Horvath-Szanics *et al*. [12], reported the presence of chitinase-coding genes, as well as chitin-binding proteins, in only five out of 43 strains from 12 different Lactobacillus species, but unfortunately, the authors were not able to detect any chitinase activity. They, therefore, concluded that although the chitin-binding proteins attach the bacteria to the chitin substrate to promote the chitinase enzyme degrading effect, these proteins could be present without any chitinase activity.

In a previous study performed by our research team [1], a LAB strain was isolated from Egyptian soil samples and identified as *Enterococcus faecium* MN32 (Gene bank accession number MH507191). The isolated strain was incubated under static conditions in the synthetic MRS broth medium (pH 6.5) at 37°C for 24 h in a static incubator. At the end of the incubation period, the clear supernatant was found to possess an antifungal ability that was demonstrated, both *in vitro* and *in vivo*, against the phytopathogen *Fusarium solani phaseoli*.

Noteworthy, *E. faecium* was formerly reported to exhibit low survival when subjected to adverse conditions [15], and this might limit its actual application in soil. Nonetheless, this drawback could be simply surmounted via encapsulating the *E. faecium* cells. On one occasion, the viability of the *E. faecium* was increased during storage and when subjected to simulated gastrointestinal conditions after its encapsulation within whey protein concentrate and native agave fructans [15]. The viability of *E. faecium* in simulated gastrointestinal fluids was also improved after the encapsulation within sodium alginate and pumpkin powder [16].

Therefore, in this study, the possible use of much cheaper cultivation media for the growth of *E. faecium* MN32 was investigated. The tested media were mainly composed of industrial and biological byproducts as alternative to the conventional MRS medium components. The consequent production of the antifungal bioagent using these media was evaluated by testing the activity of the fermented broths against the previously selected *F. solani* fungal species. Different trials were also made in an attempt to identify the nature of the produced antifungal bioagent. Finally, *E. faecium* was encapsulated in different biopolymeric matrices, and the release and the viability of the tested strain in sterile soil samples were assessed.

Materials and methods

Microorganism and culture conditions

The study was performed using a local strain of LAB, previously isolated from Egyptian soil samples and identified as *E. faecium* MN32 [1]. The cells were first of all activated by their cultivation in MRS medium [8] for 24 h at 37°C under static condition. The activated cells were then used to inoculate 12 different media, solely or partially composed of byproducts, in the final concentration of 2%. The growth of the tested strain in these media was

compared to that obtained using conventional medium (MRS medium).

Analysis of some tested byproducts

The analysis of some of the tested byproducts was performed at the Analysis and Consultation Unit, Domain of Evaluation and Remediation of Hazardous Industrial wastes, National Research Center, Dokki, Egypt. The analysis methodology was performed according to the Standard Methods for the Examination of Water and Wastewater [17] (Tables 1–3).

Treatment of some byproducts

Treatment of whey

Whey treatment was accomplished according to the method of Benaissa *et al.* [18] as follows: whey was deproteinized by heating in a water bath at 100°C for 30 min and then centrifuged at 5000 rpm for 15 min. The supernatant was collected after its filtration through a standard filter paper (DURIEUX, ref: 66301130). The treated whey contained 80% (or higher) lactose (carbohydrate) levels.

Treatment of molasses

Molasses were treated according to the method of Bae and Shoda [19] as follows: molasses were first diluted fivefold (w/v) using distilled water and then centrifuged at 600 rpm for 20 mn to separate solid materials. The supernatant was then adjusted at pH 3.0 using 4 N H_2SO_4 and heated at 100°C for 20 mn. The obtained solution was kept overnight at room temperature and then centrifuged at the same previously mentioned conditions, and the precipitates were discarded.

Table 2 Analysis of corn steep liquor

Parameters	Units	Results	Analysis methodology APHA (2017)
pH	–	4.2	4500HB
Chemical oxygen demand	gO_2/l	1600	5220D
Biological oxygen demand	gO_2/l	840	5210B
Total organic carbon	g/l	420	5310C
Total suspended solids	g/l	400	2540D
Total nitrogen	g/l	61	4500NB
Total phosphorous	g/l	1.5	4500PC
Hydrogen sulfide	g/l	0.4	4500SB
Oil and grease	g/l	17.3	5520B
Calcium, Ca	g/l		3500-Ca
Magnesium, Mg	g/l		3500-Mg
Sodium, Na	g/l	3.2	3500-Na
Potassium, K	g/l	18	3500-K
Iron, Fe	g/l	0.05	3111B
Lead, Pb	g/l	<0.001	3111B
Cupper, Cu	g/l	<0.01	3111B
Nickel, Ni	g/l	<0.001	3111B
Chrome, Cr	g/l	<0.001	3111B

Table 1 Analysis of sugarcane molasses

Parameters	Units	Results	Analysis methodology APHA (2017)
pH	–	8.5	4500HB
Chemical oxygen demand	9gO_2/l	900	5220D
Biological oxygen demand	gO_2/l	480	5210B
Total organic carbon	g/l	210	5310C
Total suspended solids	g/l	290	2540D
Total nitrogen	g/l	9	4500NB
Total phosphorous	g/l	0.7	4500PC
Hydrogen sulfide	g/l	ND	4500SB
Oil and grease	g/l	0.04	5520B
Calcium, Ca	g/l	8	3500-Ca
Magnesium, Mg	g/l	5	3500-Mg
Sodium, Na	g/l	25	3500-Na
Potassium, K	g/l	30	3500-K
Iron, Fe	g/l	0.2	3111B
Lead, Pb	g/l	<0.001	3111B
Cupper, Cu	g/l	<0.01	3111B
Nickel, Ni	g/l	<0.001	3111B
Chrome, Cr	g/l	<0.001	3111B

Table 3 Analysis of whey

Parameters	Units	Results	Analysis methodology APHA (2017)
pH	–	4.5	4500HB
Chemical oxygen demand	gO_2/l	26	5220D
Biological oxygen demand	gO_2/l	15	5210B
Total organic carbon	g/l	8	5310C
Total suspended solids	g/l	54	2540D
Total nitrogen	g/l	2	4500NB
Total phosphorous	g/l	0.5	4500PC
Hydrogen sulfide	g/l	0.38	4500SB
Oil and grease	g/l	0.19	5520B
Calcium, Ca	g/l	0.48	3500-Ca
Magnesium, Mg	g/l	0.1	3500-Mg
Sodium, Na	g/l	3.5	3500-Na
Potassium, K	g/l	1.3	3500-K
Iron, Fe	g/l	0.0003	3111B
Lead, Pb	g/l	<0.001	3111B
Cupper, Cu	g/l	<0.001	3111B
Nickel, Ni	g/l	<0.001	3111B
Chrome, Cr	g/l	<0.001	3111B

The resulting treated molasses solution was free of undesirable components, such as heavy metals and unknown compounds, which act as growth inhibitors of cell growth.

Tested media and growth conditions

In this study, five different locally supplied byproducts, including whey, sugarcane molasses, corn steep liquor (CSL), fructose high-density syrup (FHD), and high maltose concentration syrup (HMC), were tested for their ability to support significant growth of *E. faecium* strain MN32 and consequently allow potent biological agent production by the tested strain.

The growth and the antifungal activity of the tested LAB strain was studied by inoculating 12 different media (50 ml/flask), which varied in their compositions according to the byproduct added and its ratio, with cells of *E. faecium* MN32, previously activated, for 24 h in the MRS medium. The results were compared with those obtained upon the cultivation of the aforementioned activated cells in the MRS medium (Difco, Columbus, Ohio, USA), which was used as the control medium. All of the inoculated flasks were cultivated at 37°C for 24 h under static aerobic conditions.

The composition of the tested media as well as that of the control medium were as follows:

Medium 1: MRS medium, which was composed of (g/l) peptone (10.0), yeast extract (5.0), meat extract (10.0), glucose (20.0), sodium acetate (5.0), ammonium citrate (2.0), K_2HPO_4 (5.0), $Na_2HPO_4.2H_2O$ (2.0), $MgSO_4.2H_2O$ (0.1), and $MNSO_4.4H_2O$ (0.05), adjusted at pH 6.5.
Medium 2: untreated whey waste, adjusted at pH 6.5.
Medium 3: treated whey waste, adjusted at pH 6.5.
Medium 4: untreated whey/yeast extract medium [17], which was composed of (g/l of untreated whey) yeast extract, 7.5; $MgSO_4.2H_2O$, 0.2; and $CaCO_3$, 15, adjusted at pH 6.5.
Medium 5: treated molasses, adjusted at pH 6.5.
Medium 6: treated molasses/yeast extract medium I, which was composed of (g/l of treated molasses) yeast extract, 7.5; $MgSO_4.2H_2O$, 0.2; and $CaCO_3$, 15, adjusted at pH 6.5.
Medium 7: treated molasses/yeast extract medium II, which was composed of (g/l) yeast extract, 7.5; $MgSO_4.2H_2O$, 0.2; $CaCO_3$, 15; and 500 ml of treated molasses, adjusted at pH 6.5.
Medium 8: CSL, diluted twofolds and adjusted at pH 6.5.
Medium 9: CSL/yeast extract medium, which was composed of (g/l) yeast extract, 7.5; $MgSO_4.2H_2O$, 0.2; $CaCO_3$, 15; and 500 ml CSL, adjusted at pH 6.5.
Medium 10: FHD, diluted twofolds and adjusted at pH 6.5.
Medium 11: FHD/yeast extract medium, which was composed of (g/l) yeast extract, 7.5; $MgSO_4.2H_2O$, 0.2; $CaCO_3$, 15; and 500 ml FHD syrup, adjusted at pH 6.5.
Medium 12: HMC, diluted twofolds and adjusted at pH 6.5.
Medium 13: HMC/yeast extract medium: composed of (g/l): yeast extract, 7.5; $MgSO_4.2H_2O$, 0.2; $CaCO_3$, 15, and 500 ml high maltose syrup, adjusted at pH 6.5.

Estimation of antifungal activity

The poisoned food technique, described by Wang *et al.* [20], was used to evaluate the antifungal activity of the different fermentation media under test, at the end of the incubation period, as compared with that of the MRS control medium. The later depended on measuring the reduction in the growth of the tested fungal strain as a result of individually adding each of the fermented broths in the fungus cultivation medium. The obtained results were additionally confirmed by measuring the final pH of fermentation media.

The experiment was performed by adding 1% (v/v) of each tested broth to 20 ml of potato dextrose agar (PDA) medium in sterile petri dishes. After solidification, 0.9-cm discs, retrieved from previously grown *F. solani* cultures for 5–7 days, were placed at the center of each plate. The radial growth of the fungus was monitored for 2–6 days of incubation at 30°C and compared with that of control dishes in which the fungal discs were added on unseeded PDA medium. The diameters of the growing mycelium were measured as soon as that of the control petri dishes nearly covered the entire area of the plates. The percentage of growth inhibition (I%) and the corrected inhibition (IC%) were calculated using the following formulas:

$$I\ (\%) = \left[(C - T)/C\right] \times 100.$$

$$IC\ (\%) = \left[(C - T)/(C - C_0)\right] \times 100.$$

C: fungal growth diameter in control petri dishes.

T: fungal growth diameter in test petri dishes.

C_0: diameter of added fungal disc (0.5 cm).

Calculation of bacterial cell biomass

The number of viable *E. faecium* cells, and consequently possessing antifungal activity, obtained as a result of its inoculation in each of the previously mentioned fermentation medium tested, was calculated at the end of the incubation periods using the serial dilution technique followed by counting the single colonies formed on the surface of each plate. The cell biomass of the tested LAB was also determined from a previously deduced relation between the number of cells and the cells dry weight (CDW). This relation was obtained by drying the precipitate that resulted from the centrifugation of the MRS fermentation broth at the end of the incubation period and comparing the result with that of counting the number of bacterial colonies formed on the plate after applying the aforementioned serial dilution technique.

Chromatographic analysis

Chromatographic analysis, of the fermentation medium, showing the highest antifungal activity, was performed according to the methods of Zamanova *et al.* [21] and Kim *et al.* [22] as follows:

The fermentation medium 4 was first of all clarified by centrifugation under cooling at 6000 rpm for 10 mn. The resulting supernatant was then passed through a membrane filter (Sartorius, Aubagne, France, pore size 0.45 µm, Germany) and the collected cell-free supernatant was subjected to chromatographic analysis to investigate the possible substances responsible for the antimicrobial activity of the isolated *E. faecium* MN32 culture.

High-performance liquid chromatography (HPLC) analysis was performed at the Central Laboratories, National Research Center, Cairo, Egypt, using a HPLC system (Agilent 1260, Santa Clara, California, USA) equipped with a variable wavelength UV-VIS diod array detector (Dionex Corporation, Sunnyvale, California, USA) monitored at 210 nm. The sample was injected as 5 µl into an Inert Sustain AQ-C18 HP column (diameter of 4.6 mm, length of 150 mm, and inner pore diameter of 3 µm) and run for 25 mn, using 0.005 N H_2SO_4 as a mobile phase, at different flow rates according to a linear gradient as follows: 0–4.5 mn (at 0.8 ml/mn), 4.6–4.7 mn (at 1 ml/mn), 4.8–4.71 mn (at 1 ml/mn), 4.72–8.7 mn (at 1.2 ml/mn), 8.8–9 mn (at 1.3 ml/mn), 9–22 (at 1.3 ml/mn), and 23–25 mn (at 0.8 ml/min). The column temperature was maintained at 55°C.

Estimation of chitinase enzyme production ability

Cultivation of Enterococcus faecium on colloidal chitin/ agar medium

The ability of the tested microorganism to produce chitinases enzymes was evaluated by its cultivation on colloidal chitin agar medium composed of (g/l of phosphate buffer pH7) colloidal chitin, 20, and bacteriological agar, 20.

The colloidal chitin used to prepare the colloidal chitin agar medium was obtained from pure chitin (Sigma, St. Louis, Missouri, USA), as described by El-Masry [23], who followed the method of Murthy and Bleakley [24] with some modifications, as follows:

(1) To 150 ml of concentration HCl, 10 mg of pure chitin was added.
(2) The dissolved chitin was dropwise added to 2 l of cold DW.
(3) The solution was refrigerated for 18 h.
(4) The produced ppt. of colloidal chitin was centrifuged and then sterilized by autoclaving at 121°C and 15 psi for 15 min.
(5) Finally, the sterile chitin cake was converted into fine chitin powder via lyophilization.
(6) The needed amount of colloidal chitin was prepared by adding phosphate buffer of pH 7 to the equivalent amount of the chitin powder before its sterilization, along with the agar content, by autoclaving.

Estimation of total reducing sugars

The presence of chitinase enzymes in the fermentation broths of both media 1 and 4 was further investigated indirectly by measuring the concentration of reducing sugars that would result from chitin degradation as follows:

(1) Clear fermentation broths, of either media 1 or 4, were obtained after centrifugation of the bacterial cells at 600 rpm.
(2) Aliquots of 3 ml, of both fermented media 1 and 4, were added separately to equal amounts of a colloidal chitin suspension in phosphate buffer at pH 7 (samples 1 and 2, respectively).
(3) Moreover, same aliquots of the same volume of 3 ml of both fermented media 1 and 4, were added separately to equal volumes of phosphate buffer at pH 7 (without colloidal chitin addition) to measure the amount of reducing sugars still present in the fermentation broths, therefore not resulting in colloidal chitin degradation (controls 1 and 2, respectively).

(4) The last test was repeated for the same volumes of uninoculated media 1 and 4 to measure the initial amount of reducing sugar that was present in the unfermented media (controls 3 and 4, respectively).
(5) Finally, another aliquot of 3 ml of phosphate buffer at pH 7 was added to an equal volume of the same colloidal chitin suspension to confirm the consistency of colloidal chitin in the absence of any of the tested fermentation media (control 5).
(6) After 1 h of incubation, the reaction between the chitinase enzymes assumed to be present in the fermentation broths and the chitin substrate was stopped using boiling. This step was therefore applied for all of the tested samples.
(7) All suspensions were then centrifuged at 600 rpm to discard any residual colloidal chitin.
(8) The collected supernatants were then diluted fivefolds, and 1 m of each reaction mixture was subjected to the Somogyi method [25] to measure the amount of reducing sugar in each of the aforementioned reaction mixtures.
(9) The total amounts of reducing sugars released as a result of the degradation of colloidal chitin by the produced chitinase enzymes in the tested fermentation media, as well as those present in the fermentation media, were colorimetrically measured via detecting the optical density (absorbance) of the samples, using a spectrophotometer adjusted at 520 nm, and the final optical densities of the reducing sugars, released by the effect of the produced chitinases enzymes, were deduced.
(10) The produced chitinase enzyme activities, defined as the 1 μmole of N-acetylglucoseamine released in the reaction mixture per 1 h of reaction, were finally calculated according to a previously performed standard curve using pure N-acetylglucoseamine.

Encapsulation

Different biopolymer solutions were prepared to encapsulate the *E. faecium* cells. These were soy protein isolate-alginate (SPI-Alg), gellan gum (GG), and carboxymethyl cellulose (CMC) solutions. As for the cell suspension, the *E. faecium* was propagated on the treated whey media, as previously described, and the cells were collected via centrifugation.

The SPI-Alg beads were prepared as follows:

(1) A 15% SPI solution was prepared and its pH was adjusted to 9. Afterward, it was placed in a well-sealed container and was thermally denaturated via heating in an 80°C water-bath for 45 min.
(2) The SPI solution was then stored overnight in the fridge.
(3) A 5% Alg solution was prepared and sterilized via autoclaving.
(4) Afterward, 15 ml of the SPI solution was aseptically mixed with 4 ml of the Alg solution, 0.5 ml sterile distilled water, and 0.5 ml of the *E. faecium* cell suspension.
(5) From this mixture, 5 ml was then extruded, through a syringe needle, into a jar containing 20 ml of sterile $CaCl_2$ solution to acquire the gel beads [26,27].
(6) Finally, the obtained gel beads were suspended in 20 ml of sterile DW and mixed with 20 g of sterile soil in a sterile airtight 200-ml glass jar.

The GG disc was prepared as follows:

(1) A 4% GG solution was prepared and sterilized via autoclaving.
(2) To 15 ml of warm GG solution, 4.5 ml of sterile DW and 0.5 ml of cell suspension were added and thoroughly mixed so that the final concentration of GG was 3% as this was the concentration recommended by Wahba [28].
(3) Five milliliters of the GG mixture was then immediately poured into a sterile, airtight 200-ml glass jar in the bottom of which a sterile 4×4 cm piece of absorbent chromatography paper was previously placed.
(4) The GG was left to solidify, under cooling, onto the chromatography paper, which served as a support to facilitate the handling of the GG gel disc.
(5) Finally, 20 g of sterile soil and 20 ml of sterile DW were added to the jar which contained the GG disc supported on the aforementioned chromatography paper.

The CMC beads were obtained as follows:

(1) A 4% CMC solution was prepared and sterilized via autoclaving.
(2) Fifteen milliliters of the sterile CMC solution was aseptically mixed with 4.5 ml of sterile DW and 0.5 ml of cell suspension.
(3) Five milliliters of this mixture was then extruded into a jar that contained 20 ml of sterile $FeCl_3$ solution to acquire the gel beads [29].
(4) The beads remained in the gelling solution for at least 3 h before they were thoroughly washed with distilled water.

(5) Finally, 20 ml of sterile water and 20 g of sterile soil were added to a sterile airtight 200-ml glass jar along with the washed gel beads.

All of the aforementioned samples were prepared in duplicates and stored at room temperatures. At specified intervals, after gentle homogenization, 100 µl of the solution present in each jar was withdrawn and was serially diluted to assess the number of CFU in it.

A similar soil sample that contained the same amount of the free *E. faecium* cells was also prepared on equal volume basis for comparative purposes, where 0.5 ml of the cell suspension was mixed with 19.5 ml of sterile DW and poured into a sterile airtight 200-ml glass jar that contained 20 g of sterile soil.

Scanning electron microscope examination

Some SPI-Alg beads and GG discs were prepared and loaded with the *E. faecium* cells as mentioned before. These matrices were then directly placed in a 3% glutaraldehyde solution and lyophilized. Moreover, the loaded SPI-Alg beads and GG discs, which were incubated in the soil for a prolonged period of 71 days, were also processed with glutaraldehyde and lyophilized. Afterward, the surfaces of the four aforementioned gel matrices were compared via scanning electron microscopy at the Electron Microscope Laboratory, Central Laboratories, National Research Centre, Cairo, Egypt, using a QUANTA FEG-250 scanning electron microscope, under high vacuum mode.

Results and discussion

Effect of different byproducts on the growth and antifungal activity of *Enterococcus faecium* MN32

The results in Table 4 show that the *E. faecium* MN32 was able to grow on all of the tested media, even those solely composed of byproducts without any additives. Moreover, the results also confirm the ability of all of the resulting fermentation broths, of all the tested media, to highly reduce the growth diameter of the *F. solani* central disc by different percentages that ranged from about 39 to 68%, which correspond to the corrected inhibitions of about 41 to 72%. These plates were compared with the control plate, where the same disc of the fungus was added at the center of a potatodextrose agar medium, free of any added fermentation broth, and which resulted in a growth diameter of 8.5 cm.

These results were further confirmed as the measured final pHs were all found to shift toward the acidic range, thus proving the production of the different organic acids responsible for altering the growth of the tested fungal strain.

Table 4 Effect of different byproducts on the growth and antifungal activity of *Enterococcus faecium* MN32

Medium number	Medium name	Final pH	CDW (g/l)	Growth diameter (cm)	I%	IC%
1	MRS	4.2	2.4	2.7	68.2	72.5
2	Untreated whey	4.9	1.7	4.5	47	50
3	Treated whey	5.1	0.9	5.2	38.8	41.25
4	Untreated whey/yeast extract	4.3	2.3	2.8	67	71.25
5	Treated molasses	5.4	1.7	4.2	47	50
6	Treated molasses/ yeast extract I	5.9	2.0	3.45	59.4	63
7	Treated molasses/ yeast extract II	5.0	2.1	3.25	61.7	65.6
8	CSL	5.4	1.4	4.95	41.76	44.37
9	CSL/yeast extract	5.6	2.2	3.4	60	63.7
10	FHD	5.2	1.5	5.1	40	42.5
11	FHD/yeast extract	5.3	2.0	3.4	60	63.7
12	HMC syrup	5.3	1.2	5.25	38.2	40.6
13	HMC syrup/yeast extract	5.4	1.9	3.5	58.8	62.5

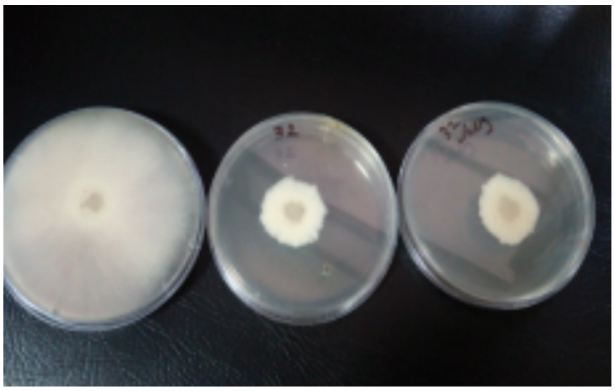
CSL, corn steep liquor; FHD, fructose high-density syrup; HMC, high maltose concentration syrup; MRS, De Man-Rogosa-Sharpe.

However, the use of medium 4, containing untreated whey along with some other components especially yeast extract in moderate amounts, particularly resulted in the highest *E. faecium* cell biomass of 2.3 g/l, the greatest inhibition % for the growth of the *F. solani* central disc of 67% and in the lowest pH of 4.3. These results were more or less similar to those obtained upon using the much more expensive MRS synthetic media used as control (Table 4, Fig. 1).

Comparative study of whey containing fermentation media

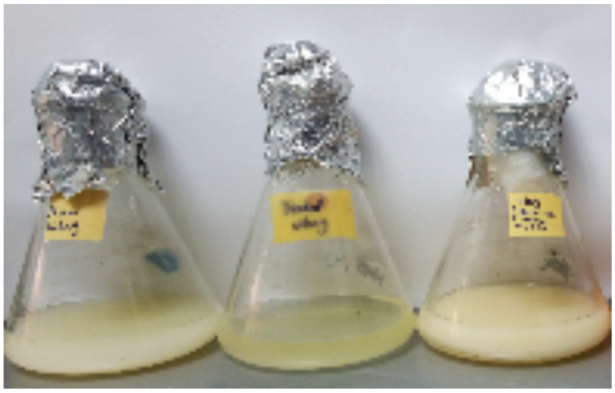
Although whey was added to media 2, 3, and 4 in equal amounts (Fig. 2), only medium 4 showed such improved results. Therefore, as an attempt to clarify the difference in the results obtained upon using media 2, 3, and 4, all containing either treated or untreated whey, either solely or among other constituents, the amount of whey added to the fermentation medium 2 was further analyzed and compared with the analysis of

Figure 1



Antifungal activity of *Enterococcus faecium* MN32 against treated *Fusarium solani* compared with untreated growth of the same fungal strain.

Figure 2



From left to right, whey-based media 2, 3, and 4, respectively.

Table 5 Comparative study of whey containing fermentation media

Tests	Medium 2	Medium 3	Medium 4
Total protein (g/100 g)	1.68	0.15	2.06
Total lactose (g/100 g)	4.38	4.41	5.76
Total solids (g/100 g)	5.55	4.14	6.10
Total ash (g/100 g)	0.69	0.69	1.28

the deproteinized whey added to medium 3 as well as with that of the total constituents of medium 4, and the results are illustrated in Table 5. These analyses were performed at the Food Safety and Quality Control Laboratory, Faculty of Agriculture, Cairo University.

The results in Table 5 reveal that the highest amounts of protein, carbohydrates (lactose), and total solids are found in medium 4. This could be explained by the fact that this medium differs from medium 2, as it contains, in addition to the raw whey, which is rich in lactose and protein, a satisfactory amount of yeast extract, which is a high-quality protein that contains a mixture of amino acids highly necessary for bacterial growth and synthesis of secondary products.

Table 6 Concentrations of organic acids detected in the fermentation broth of medium 4 using high-performance liquid chromatography

Organic acid detected	Area	Concentration (ug/ml)	Concentration (mg/ml)
Acetic acid	1403.86	6468.92	6.47
Butyric acid	5.42	47.85	0.05
Citric acid	299.09	1006.41	1.01
Formic acid	324.20	868.87	0.87
Lactic acid	1693.53	7551.45	7.55
Oxalic acid	258.26	68.38	0.07
Propionic acid	115.35	765.25	0.77
Succinic acid	742.33	4645.56	4.65

On the contrary, medium 3 contains the treated whey, which is almost completely free of protein and contains only 80% of its natural lactose content.

Investigation of the nature of the bioactive material produced

High-performance liquid chromatography of the selected fermentation broth

The results of the chromatographic analysis of the culture filtrate of the selected medium 4 (Table 6, Fig. 3) proved the presence of different organic acids, mainly including lactic and acetic acids, in the concentrations of about 7.6 and 6.5 mg/ml, respectively; succinic acid in a lower concentration of about 4.7 mg/ml; as well as other organic acids in much lower concentrations. The concentrations of all of these acids were calculated using calibration curves performed using their standard solutions.

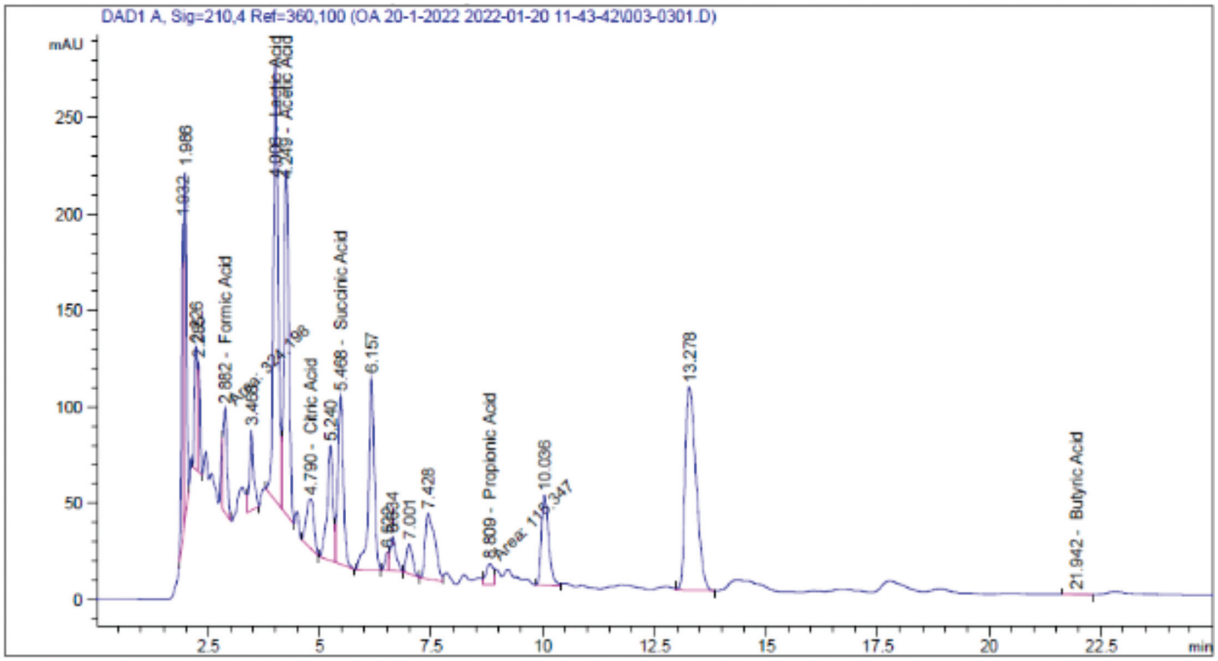
The HPLC analysis results were therefore considered as a verification for the presence of organic acids in the fermentation broth of *E. faecium* MN32 culture filtrate and this could explain its negative effect on the growth of the tested fungal strain as these acids were proven to efficiently inhibit the growth of many phytopathogenic fungi, including some *Fusarium* sp., by many investigators such as Fumagalli [30], Hassan *et al.* [31], and Guimarães *et al.* [32].

Investigation of the production of chitinases enzymes by *Enterococcus faecium* MN32

Cultivation of Enterococcus faecium MN32 on colloidal chitin

The result illustrated in Fig. 4 confirmed the ability of *E. faecium* to grow on the surface of the colloidal chitin medium thus proving its ability to consume chitin as a sole source of nutrient and consequently establishing a

Figure 3



HPLC chromatogram of the fermentation broth of medium 4. HPLC, high-performance liquid chromatography.

Figure 4

Cultivation of *Enterococcus faecium* MN32 on the surface of the colloidal chitin medium.

very high possibility that this bacterial strain does produce chitinase enzymes.

Moreover, the clear zones observed in the vicinity of the two holes, to which 100 µl of the fermentation broth of either medium 1 or 4 was added, further confirm the presence of chitinase enzymes imperatively responsible for the degradation of chitin causing the appearance of these clear zones.

This assumption could therefore explain the previously proved effect of the *E. faecium* MN32 on the growth of the tested *Fusarium* sp. (Fig. 5).

Investigation of the presence of chitinase enzymes in the fermentation broths

The results in Table 7 revealed the presence of substantial amounts of reducing sugar in the reaction mixture of both samples 1 and 2, containing fermentation broths of medium 1 and 4, respectively, which exceeded by far those present in all of the tested control test tubes. These results thus confirmed the presence of chitinase enzymes in these two tested fermentation media.

Moreover, chitinase enzymes are generally reported as inducible enzymes [33], whose production from the bacterial strain is further induced by the presence of chitin substrate in the production medium. However, unlike some of the other tested media, both media 1 and 4 contained yeast extracts, which included chitin in the cell walls of the constituting yeast cells and which may have, in addition to the apparently more convenient sugar contents of the media, boosted the production of chitinase by the *E. faecium* cells. Moreover, the yeast extract was proved to include

Figure 5

Effect of culture filtrate in the two wells on the opacity of the surrounding chitin medium.

Table 7 OD results measured at 520 nm for investigating the presence of reducing sugar in samples originating from the fermentation broths of media 1 and 4 as compared with the control

Sample/ control number	Optical density of sample (λ=520 nm)	Optical density of control (λ=520 nm)	Corrected optical density	Chitinase activity in sample (U/ reaction mixture)
1	0.9	0.65	0.25	19.5
2	1.3	1.1	0.2	18
3	–	2	2	–
4	–	1.86	1.86	–
5	–	0	0	–

Table 8 CFU/100 µl for the free and encapsulated cells

Days	Free CFU/ 100 µl $\times 10^8$	SPI-Alg CFU/ 100 µl $\times 10^8$	GG CFU/ 100 µl $\times 10^8$	CMC CFU/ 100 µl
0	1.71	0	0	0
1	1.89	0	0	0
8	1.83	0.000015	0.000007	0
15	98	0.000037	0.000038	0
22	103	0.39	0.000051	0
29	148	0.5	0.45	0
36	159	0.98	0.79	300
43	103	1.62	1.01	1100
50	98	8800	5000	2900
57	70	11 500	7600	20 000
64	57	15 800	10 500	42 000
71	47	18 900	13 300	59 000

CMC, carboxymethyl cellulose; GG, gellan gum; SPI-Alg, soy protein isolate-alginate.

some hormonal regulators that have the ability to activate the production of certain enzymes by the cultivated bacterial strain [34,35].

These findings could explain the improved antifungal activities of all of the tested media to which yeast extract was added in general, and that of fermentation medium 2 and 4 in particular, compared with the other media tested (Table 4).

Encapsulation

Table 8 revealed that at the zero time and after the first 24 h of incubation, no *E. faecium* cells were detected in the solution around the gel beads and discs, which confirmed a 100% encapsulation efficiency as all of the cells were successfully encapsulated within the gel matrices. However, on the eighth day, and after 1 week of incubation, the release of *E. faecium* cells from both the SPI-Alg beads and the GG discs, to the surrounding environment, was noticed. On the contrary, it took 36 days for the *E. faecium* cells to escape from the CMC beads. This delay could be attributed to the negative effect imparted by the $FeCl_3$ solution that was utilized to prepare the CMC beads. Ferric in the form of Fe_2O_3 nanoparticles was formerly reported to exert a bacteriostatic effect on another *Enteroccocus* strain (*Enterococcus faecalis*) [36]. The negative effect imparted by the $FeCl_3$ solution could also be the reason for the delayed release of a lower number of *E. faecium* cells from the CMC beads as the cells in this case were not detected during the first month of incubation and only 59 000 CFU/100 µl was detected at the end of the experiment, after 71 days of incubation, compared with 13 300×10^8 and 18 900×10^8 CFU/100 µl, which were observed around the GG discs and SPI-Alg beads, respectively, after the same period of time.

Moreover, comparing the amount of *E. faecium* cells recorded in the soil inoculated with the free microorganism with those detected in the vicinity of

the encapsulated cells in both SPI-Alg beads and GG discs revealed that the encapsulation increased the viability of the cells.

Moreover, the amount of *E. faecium* cells released from both encapsulation matrices was found to increase progressively with time, whereas the amount of free *E. faecium* cells started to decline on the 50th day. These results proved that these encapsulation matrices protected the cells and increased their viability [15,16].

Furthermore, it could be stated that the GG polysaccharide present in the GG discs, as well as the Alg polysaccharide and SPI protein contents of the SPI-Alg beads, could have provided the *E. faecium* cells in these matrices, especially the SPI-Alg beads which contained both nutrient sources, with additional nutrients that further boosted their growth and viability.

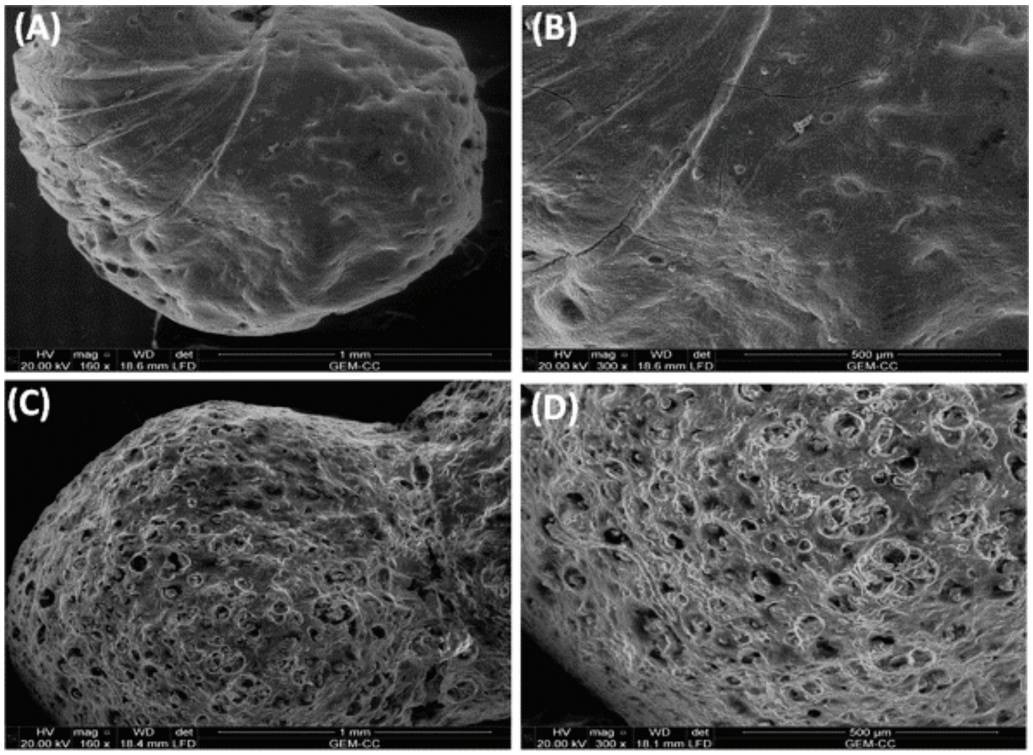
However, the results of Table 8 also revealed that, at the end of the experiment, the number of cells released from the SPI-Alg beads, highly exceeded those released from the GG discs, and this could be attributed, in addition to the presence of protein in the former matrix, to the much higher surface area of the SPI-Alg beads when compared with that of the GG discs.

These findings therefore confirmed that the encapsulation of the tested strain within SPI-Alg beads was the most effective technique that could be used to ensure the sustained release of adequate numbers of *E. faecium* cells, previously proven to possess an efficient antifungal activity, to the agricultural soil.

Scanning electron microscope examination of some gel beads and discs

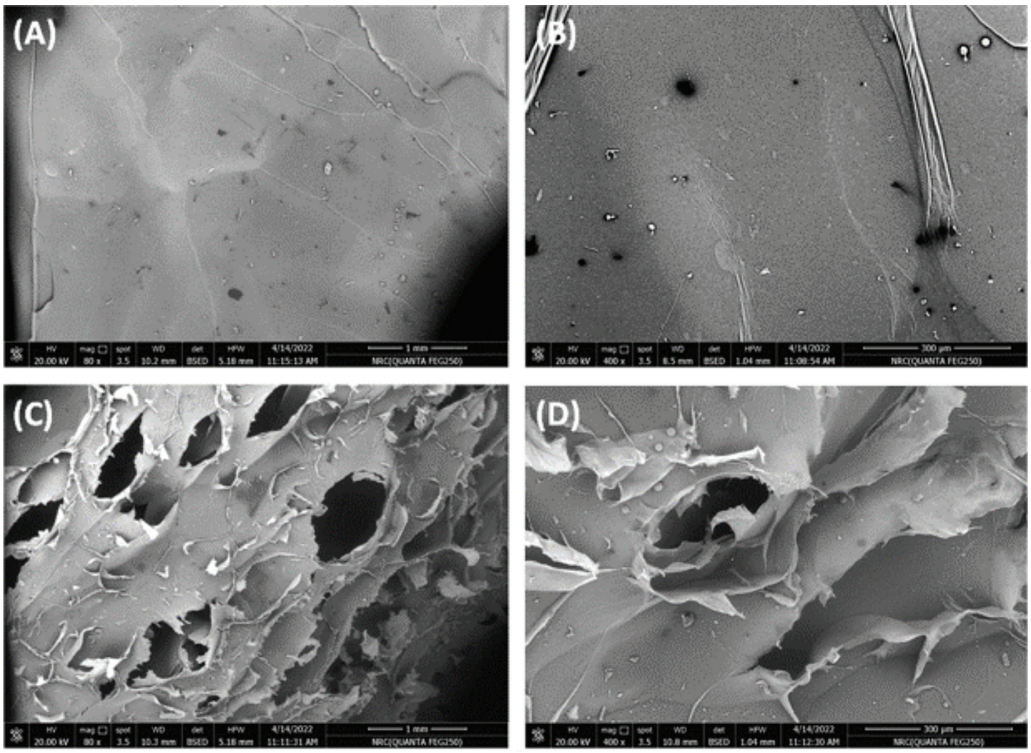
The feeding of *E. faecium* cells on the SPI-Alg and GG matrices could be confirmed from the scanning electron microscope images, which explored the surfaces of the freshly prepared gels in comparison with that of the same gels at the end of the incubation period (Figs 6 and 7). The images clearly revealed that the surfaces of both the loaded SPI-Alg beads and GG discs contained obvious pores after the prolonged period of incubation, thus proving that the *E. faecium* cells fed on the polymeric matrices and created pores, which allowed for the increased release of large numbers of the *E. faecium* cells.

Figure 6



Surface morphologies of loaded SPI-Alg beads either freshly prepared (a–b) or after 71 days of incubation in soil (c–d) at ×160 (a–c) and ×300 (b–d) magnification. SPI-Alg, soy protein isolate-alginate.

Figure 7



Surface morphologies of loaded GG discs either freshly prepared (a–b) or after 71 days of incubation in soil (c–d) at ×80 (a–c) and ×400 (b–d) magnification. GG, gellan gum.

Conclusion

The results of the performed experiments revealed that *E. faecium* MN32, previously isolated from fertile Egyptian soil and molecularly identified as a LAB, is significantly active against *F. solani*, which is a fungal phytopathogen that consistently affects economically important crops, by the production of safe and effective bioagents such as some organic acids and chitinase enzymes. Furthermore, the results also demonstrated that the isolated LAB could be successfully grown on a number of natural byproducts, especially whey, which are considered as relatively cheap media as compared with the more expensive synthetic media such as the MRS medium. It was also concluded that encapsulating *E. faecium* within SPI-Alg beads or GG discs increased the viability of the cells in soil samples.

Accordingly, it could be stated that the application of these produced safe, economic encapsulated gel beads in crop control could be a very promising approach to reduce not only the losses in agricultural crops but also the extensive use of hazardous chemicals and toxic pesticides.

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Conflicts of interest

There are no conflicts of interest.

References

1 Atwa NA, El-Diwany AI, Daba GM, Elmaghraby IMK, Elmaghraby MMK, Amer MN, Maany DA. Studies on the antimicrobial activity of a locally isolated lactic acid bacterium and its application on *Fusarium solani*-infected plants. Egypt Pharma J 2021; 20:207–220.

2 Klaenhammer TR. Bacteriocins of lactic acid bacteria. Biochimie 1988; 70: 337–349.

3 Ouwehand A, Vesterlund S. Lactic acid bacteria: microbiology and functional aspects. In: Salimen S, Von Wright A, Ouwehand A, editors.

Antimicrobial components from lactic acid bacteria. 3rd ed. New York, USA: Marcel Dekker 2004; 139: 375–396.

4 Tagg RJ, Mc Govern RA. Assay system for bacteriocins. Appl Microbiol 1971; 21: 943.

5 Hitha CS, Hima CS, Yogesh BJ, Bharathi S, Sekar KV. Microbial utilization of dairy waste for lactic acid production by immobilized bacterial isolates on sodium alginate beads. Int J Pure Appl Biosci 2014; 2: 55–60.

6 Bouson S, Krittayavathananon A, Phattharasupakun N, Siwayaprahm P, Sawangphruk M. Antifungal activity of water-stable copper-containing metal-organic frameworks. R Soc Open Sci 2017; 4: 170654.

7 Mathur H, Beresford TP, Cotter PD. Health benefits of lactic acid bacteria (LAB) fermentates. Nutrients 2020; 12: 1679–1695.

8 De Man JC, Rogosa M, Sharpe ME. A medium for the cultivation of Lactobacilli. J Appl Bacteriol 1960; 23: 130–13.

9 Özer B, Kirmaci H. Functional milks and dairy beverages. Int J Dairy Technol 2010; 63: 1–15.

10 Palmonari A, Cavallini D, Sniffen CJ, Fernandes L, Holder P, Fagioli L, *et al.* Characterization of molasses chemical composition. J Dairy Sci 2020; 103: 6244–6249.

11 Patel S, Goyal A. Chitin and chitinase: role in pathogenicity, allergenicity and health. Int J Biol Macromol 2017; 97: 331–338.

12 Horvath-Szanics E, Perjéssy J, Klupács A, Takács K, Nagy A, Koppány-Szabó E, *et al.* Study of chitinase and chitinolytic activity of lactobacillus strains. Acta Aliment 2020; 49: 214–224.

13 Leisner JJ, Vogensen FK, Kollmann J, Aideh B, Vandamme P, Ingmer H. α-chitinase activity among lactic acid bacteria. Syst Appl Microbiol 2008; 31: 151–156.

14 Beck HC, Madsen SM, Glentino J, Petersen J, Israelsen H, Hansen AM. Proteomic analysis of cell surface-associated proteins from probiotoc *Lactobacillus plantarum*. FEMS Microbiol Lett 2009; 297: 61–66.

15 Cancino-Castillo LA, Beristain CI, Pascual-Pineda LA, Ortiz-Basurto RI, Juárez- Trujillo N, Jiménez-Fernández M. Effective microencapsulation of *Enterococcus faecium* in biopolymeric matrices using spray drying. Appl Microbiol Biotechnol 2020; 104: 9595–9605.

16 Nguyen TH, Kim YG, Kim JS, Jeong Y, Park HM, Kim JW, *et al.* Evaluating the cryoprotective encapsulation of the lactic acid bacteria in simulated gastrointestinal conditions. Biotechnol Bioprocess Eng 2020; 25: 287–292.

17 APHA Standards methods for the examination of water and wastewater. 23rd ed. Washington, DC: American Public Health Association (APHA); 2017.

18 Benaissa M, Zadi-Karam H, Karam NE. Development of a sweet whey-based medium for culture of Lactobacillus. Afr J Biotechnol 2017; 16: 1630–1637.

19 Bae S, Shoda M. Production of bacterial cellulose by *Acetobacter xylinum* BPR 2001 using molasses medium in a jar fermentor. Appl Microbiol Biotechnol 2005; 67: 45–51.

20 Wang H, Yan H, Shin J, Huang L, Zhang H, Qi W. Activity against plant pathogenic fungi of Lactobacillus plantarum IMAU10014 isolated from Xinjiang koumiss in China. Ann Microbiol 2011; 61: 879–885.

21 Zamanova M, Glotova V, Izhenbina TN, Krutas D, Novikov VT. Simultaneous HPLC-UV determination of lactic acid, glycolic acid, glycolide, lactide and ethyl acetate in monomers for producing biodegradable polymers. Proc Chem 2014; 10:244–251.

22 Kim HJ, Lee MJ, Kim HJ, Cho SK. Development of HPLC-UV method for detection and quantification of eight organic acids in animal feed. J Chromatogr Separat Tech 2017; 8:1–5.

23 El-Masry HM. Eco-friendly microbial biosynthesis of chitinases and formation of N-acetylglucoseamine [MSc thesis] Ismailia, Egypt: Faculty of Science, Suez Canal University, 2017.

24 Murthy N, Bleakley B. Simple method of preparing colloidal chitin used for screening of chitinase-producing microorganisms. Internet J Microbiol 2012; 10: 1–4.

25 Somogyi MJ. Notes on sugar determination. J Biol Chem 1952; 195: 19–23.

26 Elnashar MMM, Wahba MI, Amin MA, Eldiwany AI. Application of Plackett-Burman screening design to the modeling of grafted alginate-carrageenan beads for the immobilization of penicillin G acylase. J Appl Polym Sci 2014; 131: 11.

27 Abaee A, Mohammadian M, Jafari SM. Whey and soy protein-based hydrogels and nano-hydrogels as bioactive delivery systems. Trends Food Sci Technol 2017; 70: 69–81.

28 Wahba MI. Processed gellan gum beads as covalent immobilization carriers. Biocat Agr Biotechnol 2018; 14: 270–278.

29 Huei GOS, Muniyandy S, Sathasivam T, Veeramachineni AK, Janarthanan P. Iron cross-linked carboxymethyl cellulose-gelatin complex coacervate beads for sustained drug delivery. Chem Pap 2016; 70: 243–252.

30 Fumagalli C. Succinic acid and succinic anhydride. In: Kroschwitz JI, Howe-Grant M, editors. Kirk-Othmer encyclopedia of chemical technology. 4th ed. New York, USA: Wiley 1997; 22: 1074–1102.

31 Hassan R, El-Kadi S, Sand M. Effect of some organic acids on some fungal growth and their toxins production. Int J Adv Biol 2015; 2: 1.

32 Guimarães A, Venancio A, Abrunhosa L. Antifungal effect of organic acids from lactic acid bacteria on *Penicillium nordicum*. Food Addit Contam A Chem Anal Control Expo Risk Assess 2018; 35: 1803–1818.

33 Poria V, Rana A, Kumari A, Grewal J, Pranaw K, Singh S. Current perspectives on chitinolytic enzymes and their agro-industrial applications. Biology 2021; 10: 1319.

34 Horinouchi S, Beppu T. Regulation of secondary metabolism and cell differentiation in *Streptomyces*. A-factor as a microbial hormone and the AfsR protein as a component of a two component regulatory system. Gene 1992; 115: 167–172.

35 Horinouchi S, Beppu T. A-factor as a microbial hormone that controls cellular differentiation and secondary metabolism in *Streptomyces griseus*. Mol Microbiol 1992; 12: 859–864.

36 Shahbazi E, Morshedzadeh F, Davood Zaeifi D. Bacteriostatic potency of Fe_2O_3 against Enterococcus faecalis in synergy with antibiotics by DDST method. Avicenna J Med Biotech 2019; 11:176–179.