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Enhancement Productivity of Lactic acid bacteria (LAB) to Produced Expolysaccharide

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ABSTRACT

Isolation and characterization of exocellular polysaccharide was studied in order to evaluate some parameters in the synthesis of exopolysaccharide (EPS) and improve their production through submerged fermentation processes. Isolation strains *Lactobacillus delbrueckii ssp bulgaricus* (**IS₁**), *Lactococcus lactis ssp cremoris* (**IS₂**) and *Lactobacillus delbrueckii ssp bulgaricus* (**IS₃**) were studied in shake flasks using yeast extract, surfactants and different exposure doses of gamma irradiation. The optimum concentration of (EPS) formation (0.762 g/l) by *Lactococcus lactis ssp cremoris* (**IS₂**) at and 3.0(g/l) yeast extract, 1.72 (g/l) at 0.5 (%) surfactant Triton X-100. Also, EPS (1.842 g/l) was produced when *Lactococcus lactis ssp cremoris* (**IS₂**) exposed to 0.2 kGy dose level.

INTRODUCTION

Many strains of lactic acid bacteria (LAB) produce exopolysaccharides (EPSs), which can be either attached to the bacterial surface as a capsular polysaccharide (CPS) or secreted into the surrounding medium as a slime polysaccharide (SPS). Most EPS-producing LA strains reported to date produce SPS, while some LAB strains can simultaneously produce both CPS and SPS ⁽¹⁾. EPSs produced by LAB have received increased attention mainly because these biopolymers are able to improve the rheological properties of fermented milk ⁽²⁾, and they may function as natural alternatives to commercial stabilizers ⁽³⁾. Some EPSs may also possess immunostimulatory and antitumor activities ^(4, 5). It has been shown that LAB cultures producing slime and/or capsular EPSs can improve the texture of fermented milk ⁽⁶⁾ and increase the moisture retention of cheese ^(7, 8). The use of EPS-producing strains of *Streptococcus thermophilus* can reduce the level of syneresis and gel firmness of set type yogurt ⁽⁹⁾. The physical properties of yogurt can be improved by the use of EPSs produced in situ or added as bioingredients ⁽¹⁰⁾. EPS–EPS and EPS–protein interactions in fermented milk might promote the formation of a complex network gel structure, beneficially affecting the rheological behavior of the product, as shown by microstructural studies of EPS-producing lactic cultures ⁽¹¹⁾. Among EPS-producing LAB, *Lactobacillus rhamnosus* strains have been reported to produce EPSs varying in amount, monosaccharide composition and EPS structure. *L. rhamnosus* GG (ATCC 53103), a wellknown probiotic strain, has been shown to produce an EPS containing galactose, rhamnose and N-acetylglucosamine in a molar ratio of 4:1:1 ⁽¹²⁾ and also a capsular polysaccharide (Huttunen, E., and Alatosava, T., Poster, YAKULT symposium, Finland, 2005). *L. rhamnosus* strain C83 produces an EPS mainly composed of galactose and glucose, and the sugar composition of this polymer was shown to be independent of culture conditions ⁽¹³⁾. *L. rhamnosus* RW-9595M was reported to produce

an EPS in a high yield (1,808 mg/L), and it was shown to be an effective biothickener with potential EPS-linked healthful properties⁽¹⁴⁾. *L. rhamnosus* strains ATCC 9595, R, RW-9595M and RW-6541M have been shown to produce different amounts of EPSs but with little variation in the gene clusters encoding EPS production⁽¹⁵⁾. The aim of this study was to find EPS-producing LAB strains potentially applicable in the dairy industry for improving the physical properties of fermented milk products. LAB strains were isolated from homemade Chinese sauerkraut, a naturally fermented product where EPS-producing bacteria might survive under the unfavorable growth conditions, i.e., high acidity and salinity, due to the protective nature of the polymers for the cells⁽¹⁶⁾. Of the EPS-producing LAB strains screened, one strain was identified as *L. rhamnosus*. Here, we report the isolation and identification of LAB strain and show evidence for the production of EPS by this strain.

MATERIALS AND METHODS

Sampling

Thirty samples of fermented milk were obtained from individual households in rural areas in northern Burkina. Samples were collected in sterile small bottles and stored in laboratory under refrigeration at 4°C until they were used in experiments.

Isolation of lactic acid bacteria

Serial dilutions of homogenized fermented milk samples in 0.1% peptone saline were used for microbial isolation with the following media: (a) MRS agar⁽¹⁷⁾ (Fluka Biochemika 69966) incubated anaerobically for 48 h at 42°C for isolation of thermophilic *Lactobacilli* and *Streptococci*, (b) MRS agar incubated anaerobically for 48 h at 35°C for isolation of mesophilic *Lactobacilli* and *Leuconostoc*, (c) M17 agar⁽¹⁸⁾ (Difco) incubated aerobically for 48 h at 30°C for isolation of *lactococci*, (d) Rogosa agar (Difco) incubated anaerobically for 48 h at 35°C for isolation of *lactobacilli*. Fifty isolates were obtained by random selection of slimy (exopolysaccharides producer) colonies from all media used. These isolates were tested for exopolysaccharides production in modified MRS broth.

Phenotypic and biochemical characterization of selected isolates

Gram staining, catalase activity, gas production from glucose, growth in NaCl 6.5% was determined according to methods for lactic acid bacteria. Cells morphology was determined with cells grown in MRS broth for 35°C at 20 h by using phase-contrast microscopy.

Isolation, purification, and quantification of EPS

Selected isolates were grown in modified MRS broth (glucose 20 g/l was replaced by lactose 75 g/l), cells were harvested by centrifugation for 10 min at 11 000 x g. Two volumes of cold ethanol were added to culture supernatants and stored overnight at 4°C. Precipitated material was collected by centrifugation (20 min at 2500 x g) resuspended in demineralised water, and mixed with 2 volumes of cold ethanol. Samples were centrifuged 2500 x g and the pellets were dried at 100°C. The total carbohydrate content of the EPS was determined using the phenol-sulfuric acid procedure⁽¹⁹⁾.

EPS Producing Strains

EPS producing lactic acid bacterial strains including *Lactococcus lactis* ssp. *cremoris* DSM20069, *Lactobacillus delbrueckii* ssp. *bulgaricus* EMCC 1102 and *Lactobacillus rhamnosus* EMCC 1105 were obtained from Egyptian Microbial Culture Collection (EMCC) at Cairo

Microbiological Resources Center (Cairo MIRCEN), Faculty of Agriculture, Ain shams University. Additional EPS producing LAB strains were isolated from dairy products in this study.

Dairy Products Samples

Yoghurt, buttermilk and Karish cheese were collected from local markets of Cairo in 2004. The samples were kept refrigerated at 4°C until they were used for isolation of EPS producing lactic acid bacteria.

Fresh milk

Fresh cow's milk (3.25% fat, 8.54% SNF, acidity 0.153%) used for yoghurt manufacture was obtained from Dina for Agricultural Investments Co., Alexandria Desert Road.

Skim milk powder

Skim milk powder (Low Heat) used for yoghurt fortification was obtained from Dina for Agricultural Investments Co., Alexandria Desert Road.

Cheese Whey

Sweet whey (0.27% protein, 4.9% lactose, 0.5% ash and 5.8% total solids) from Gouda cheese manufacturing was obtained from Dina for Agricultural Investments Co., Alexandria Desert Road.

MRS medium

MRS medium used for LAB strains preservation, culturing, counting and EPS production was obtained from OXOID, UK.

Gamma irradiation

The effect of different doses of gamma irradiation on EPS production and properties were investigated. Irradiation process was carried out in gamma chamber 400 A (Isotope Group, Bhabha Atomic Research Center Trombay /Bombay /India) using Co⁶⁰ at dose rate 5.38 KGy/h at the time of the experiment, located at the National Center for Radiation Research and Technology (NCRRT), Nasr City, Cairo-Egypt was used for irradiation process.

Experimental procedures

Isolation of lactic acid bacterial strains with high EPS production

Fifty EPS producing strains were isolated by selective plating (from buttermilk, Kareech cheese and yoghurt) on MRS and M17 Agar and anaerobic incubation on 37 C for 72 h. EPS producing colonies were evaluated by touching colonies with a sterile inoculating loop. The presence of ropy strands between the loop and the colony as the loop was slowly raised was considered EPS positive. The colonies were picked up and propagated in MRS broth for 24 h. The EPS producing isolates were verified for gram positive and catalase negative rods and cocci. The purity of cultures were verified by spreading cultures on MRS agar and repeated several times until a pure culture was obtained. The defined and isolated lactic acid bacterial cultures were stored at - 40°C in MRS broth medium⁽²⁰⁾ plus 15% glycerol solution until required. The fifty EPS producing isolates were revived by transferring 0.5 ml of each frozen stock to 10 ml of MRS broth and incubating at 37°C for 48 h. The revived cultures were reactivated again by transferring 1.0 ml to 10 ml MRS broth and incubating at 37°C for 48 h. the reactivated cultures were used as standard inoculum culture (SIC)⁽²¹⁾.The fifty

reactivated isolates were inoculated with a ratio of 2.5% (v/v) of (SIC) into 100 ml MRS broth in 250 ml capacity conical flasks in triplicates. All flasks were incubated at 37°C for 48 h. By the end of fermentation period, the isolates were evaluated for EPS production by determine the EPS concentration (g / l) in the final fermented MRS media.

Evaluation of cheese whey for EPS production by LAB strains

In this experiment, the highest EPS producing isolates (*Lb. delbrueckii ssp. bulgaricus* I1 isolated from yoghurt, *Lc. lactis ssp. cremoris* I2 isolated from butter milk and *Lb. delbrueckii ssp. bulgaricus* I3 isolated from karish cheese), *Lactococcus lactis ssp. cremoris* DSM20069, *Lactobacillus delbrueckii ssp. bulgaricus* EMCC 1102 and *Lactobacillus rhamnosus* EMCC 1105 were used for EPS production in MRS broth and whey based medium (WBM). Sweet Gouda cheese whey was autoclaved at 121°C for 5 min to precipitate the whey protein; the precipitate was removed by filtration through Whatman paper No.1⁽²²⁾. After filtration the pH was adjusted to 6.2 with (0.1N NaOH) and then fortified with different mineral salts including: KH₂PO₄ 2g/l, NH₄CL 0.75g/l and mineral solution 4 ml/l (MgSO₄.7H₂O 10g/l, MnSO₄.7H₂O 0.7g/l, FeSO₄.7H₂O 0.4g/l, and CaCL₂.2H₂O 0.1g/l). Both MRS and WBM were used as EPS production media (EPS-PM). They were distributed in 250 ml conical flasks with a working volume of 100 ml. EPS – PM were autoclaved at 121 C for 5 min and 121 C for 15 min For WBM and MRS, respectively. EPS-PM were inoculated with 2.5 ml of (SIC) and incubated for 37° C for 80 h. At 8 h intervals, 1ml was aseptically with drawn from each flask under aseptic conditions to determine growth and production kinetics including growth intensity (OD), residual sugar (g/l), and consumed sugar (g/l) EPS production (g/l) and biomass dry weight (g/l).

Optimization of fermentation processes for EPS production by LAB strains

Yeast extract

In this experiment, EPS – PM were supplemented with yeast extract as a source for growth factors with concentrations of 1, 2 and 3 g/l.

Effect of gamma irradiation on EPS production by LAB strains

Irradiation process

The highest EPS producing strains were sub cultured in MRS medium and incubated at 37°C for 48 h at agitation speed of 50 rpm in 250 ml Erlenmeyer flasks containing 100 ml sterile medium with initial pH 6.2. The inoculum density was adjusted by measuring the optical density at 600 nm using spectrophotometer. Five milliliter of inoculum culture broth were transferred to sterile tubes and exposed for increasing doses of gamma radiation (0.0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 kGy) at ambient temperature (triplicates for each dose). The irradiated broth culture (5 ml) was used as an inoculum in the EPS-PM (triplicates for each dose) was incubated at 37°C for 48 h and 50 rpm.

D₁₀-Value

The exposed cells were serially diluted in 9ml sterile isotonic saline solution, from the appropriate dilutions, 1 ml suspension was added under MRS agar medium and incubated at 37°C for 48 hrs, and then the growing cells were counted. A dose response curve was drawn by plotting the dose (kGy) against log surviving cells.

Surfactants

In this experiment, EPS – PM were supplemented with three types of surfactants including Tween 40 and Tween 80, and triton X-100 at concentrations of 0.1, 0.25 and 0.5 %.

Methods of analysis

Isolation and identification of EPS producing LAB

The most efficient EPS producing isolates were completely identified according to BIOLOG System (GP2 Microplate). The morphological properties were shape of cells, gram staining and shape of colonies. Physiological properties were catalase reaction was determined by the 3% H₂O₂ method⁽²³⁾.

EPS Dry Weight in MRS

The EPS concentration was determined according to the method described⁽²⁴⁾.

EPS Dry Weight in cheese whey

The EPS was dried under vacuum and weighted⁽²⁴⁾.

Analytical methodes

The yield factor of growth was calculated according to the equation and Sugar utilization efficiency, Polymer (EPS) parameters were calculated according to the equations reported⁽²⁵⁾. The D₁₀ value of each strain was calculated from the dose response curve, it is the dose required to reduce the surviving cells by one log cycle. The D₁₀ value could be calculated from the equation of⁽²⁶⁾.

Total sugars were determined using the phenol sulfuric method as described⁽¹⁹⁾. The data were analyzed using one-way analysis of variance (ANOVA) by statistical analysis system (**SAS Institute, Inc.**)⁽²⁷⁾. Duncan's multiple rang test was used to compare the means when a significant variation was established by ANOVA at the significance level 0.05 (P < 0.05).

RESULTS AND DISCUSSION

Isolation of lactic acid bacterial strains with high EPS production:

In this study, fifty bacterial strains were isolated from different dairy products including traditional yoghurt (Zabady), Karish cheese and buttermilk. The primary characterization of bacterial isolates indicated that they were gram positive, rods and cocci, catalase negative and anaerobic. Data presented in Table (1) revealed that highest numbers of bacterial isolates were picked-up from yoghurt and Karish cheese followed by buttermilk being 20, 20, and 10, respectively. This could be attributed to manufacturer procedures.

All isolates were screened for EPS production in MRS broth, when they grown anaerobically at 37°C for 48 h as an EPS production period. According to their efficiency for EPS production, the isolates could be categorized into four categories namely high, moderate, weak and inactive, which produce EPS concentrations ranged from 0.05 to 0.5 g/l, 0.02 to 0.05, 0.002 to 0.02 and less than 0.002, consecutively. The major percentage of these isolates were presented in the inactive category followed by weak and moderate categories i. e. inactive, weak and moderate EPS producing isolates being 44, 32 and 18%, successively. Three out of 50 bacterial isolates (6 %) fall in the high EPS producing category (from 0.05 - > 0.5 g/l), which were isolated from yoghurt, Karish cheese and buttermilk, one from each product. Therefore, these three isolates were subjected to

complete identification using BIOLOG System. They were identified as lactic acid bacterial strains namely: *Lb. delbrueckii* ssp. *bulgaricus* I₁, *Lc. lactis* ssp. *cremoris* I₂, *Lb. delbrueckii* ssp. *Bulgaricus* I₃. It was found that these three isolates had characteristics similar to *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactococcus lactis* ssp. *cremoris*. gram positive, rods and cocci, catalase negative and anaerobic bacteria. These results are in agreement with who stated that certain strains of LAB are able to synthesize exopolysaccharides (EPS) that are secreted into their environment such as milk and yoghurt⁽²²⁾.

Table (1): Categorizes of bacterial isolates (from some dairy products) according to their EPS production.

Dairy Products	Isolates		EPS Producing Categories							
			High		Moderate		Weak		Inactive	
	No.	%	No.	%	No.	%	No.	%	No.	%
Yoghurt	20	40	1	2	5	10	8	16	6	12
Karish Cheese	20	40	1	2	1	2	6	12	12	24
Butter Milk	10	20	1	2	3	6	2	4	4	8
Total	50	100	3	6	9	18	16	32	22	44

Growth Intensity

Growth intensity as optical density (O.D) of the three bacterial isolates (*Lactobacillus delbrueckii* ssp. *bulgaricus* I₁, *Lactococcus lactis* ssp. *cremoris* I₁ and *Lactobacillus delbrueckii* ssp. *bulgaricus* I₃) and three EPS producing reference strains including *Lactococcus lactis* ssp. *cremoris* DSM 20069, *Lactobacillus delbrueckii* ssp. *bulgaricus* EMCC 1102 and *Lactobacillus rhamnosus* EMCC 1105 grown in MRS broth and Whey Based Medium (WBM) at 37 °C or 42 and 45°C (EMCC 1102 and EMCC 1105) for 80 h, is presented in Tables (4 and 5) and illustrated by Fig. (1 and 2). During the first 40 h of incubation period, growth intensity in MRS medium increased for all six tested strains. The time required to achieve maximum O.D varied from 32 to 40 h. The highest intensity (log O.D = 0.292) was recorded after 40 h by *Lactococcus lactis* ssp. *cremoris* DSM 20069 followed by *Lactobacillus delbrueckii* ssp. *bulgaricus* EMCC 1102 (log O.D = 0.289) and *Lactobacillus rhamnosus* EMCC 1105 (log O.D = 0.235). Regarding the bacterial isolates, it was observed that highest intensity (log O.D = 0.185) was recorded after 32 h by *Lactobacillus delbrueckii* ssp. *Bulgaricus* I₁ followed by *Lactococcus lactis* ssp. *cremoris* I₂ (log O.D = 0.154) and *Lb. bulgaricus* I₃ (log O.D = 0.107) were presented Fig. (1). Finally, it was observed that after 56 to 64 h of incubation period, the growth intensity in MRS medium decreased for all tested strain. Following the same trends, the data presented Fig (2) indicated that growth intensity during the first 40 h of incubation period in WBM increased. The time required to achieve maximum O.D varied from 32 to 48 h. The highest intensity (log O.D = 0.149) was recorded after 48 h by *Lactococcus lactis* ssp. *cremoris* DSM 20069 followed by *Lactobacillus delbrueckii* ssp. *bulgaricus* EMCC 1102 (log O.D = 0.102) in the same fermentation period. *Lactobacillus rhamnosus* EMCC 1105 reached the highest growth intensity (log O.D = 0.138) after 32 h. Regarding the bacterial isolates, it was observed that

highest intensity (log O.D = 0.092) was recorded after 32 h by *Lactobacillus delbrueckii ssp. bulgaricus* I₁ followed by *Lactobacillus delbrueckii ssp. bulgaricus* I₃ (log O.D = 0.087) and *Lactococcus lactis ssp. cremoris* I₂ (log O.D = 0.079). In addition, the results revealed that during 32 - 56 h incubation period, symmetric growth intensity for the strains was recorded as grown in both MRS broth and WBM. Finally, it was observed that after 56 - 64 h of incubation period, the growth intensity in WBM decreased for all tested strains. When comparing the growth intensities achieved by the tested strains in both fermentation media, it was observed that reference strains achieved higher growth intensity than bacterial isolates. Meantime, the growth intensities achieved in MRS broth were higher than these achieved in WBM.

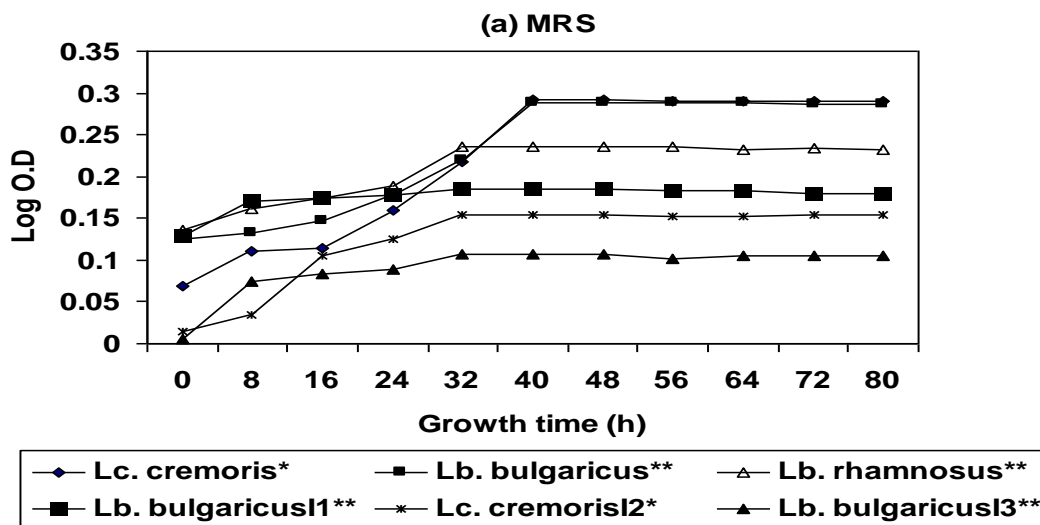


Fig (1): Growth intensity (Log O.D) of EPS producing reference strains and isolates grown in MRS broth at 37 °C or 42 °C (EMCC 1102 and EMCC 1105) for 80 h.

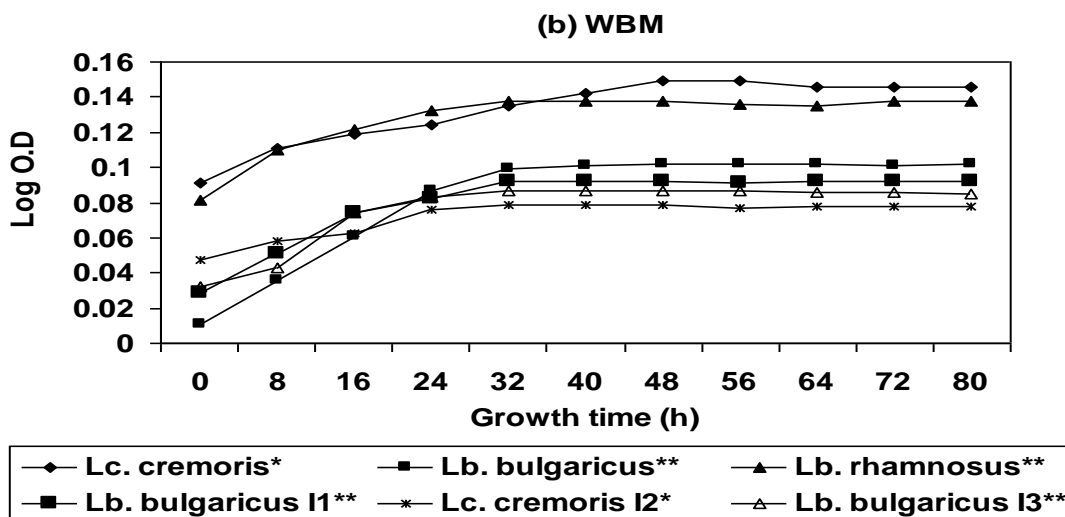


Fig (2): Growth intensity (Log O.D) of EPS producing reference strains and isolates grown in WBM broth at 37 °C or 42 °C (EMCC 1102 and EMCC 1105) for 80 h.

Specific Growth Rate and Doubling Time

Data presented in Table (2) indicate that maximum specific growth rate (μ) and minimum doubling time (t_d) were achieved in MRS broth by *Lb. bulgaricus* (0.039 and 17.8, respectively). Where are the maximum specific growth rate (μ) and minimum doubling time (t_d) recorded in WBM (0.033 and 21, respectively) were achieved by the *Lactobacillus delbrueckii ssp. bulgaricus* I₁. These observations are confirmed by the general linear model (GLM) statistical analysis as proved that there were highly significant difference ($P < 0.05$) among the (μ) and (t_d) in MRS and WBM. These results could be attributed to the difference between MRS and WBM compositions. The MRS is a synthetic medium supplemented with growth factors including mineral salts, vitamins, and amino acids. In addition, the results could be attributed to the variations in carbon sources and the different abilities of these strains to utilize the available carbon source (glucose in MRS and lactose in WBM) in the fermentation media^(28, 29).

Table (2): Specific growth rate (μ) and Doubling Time (t_d) of EPS producing reference strains and isolates grown in MRS and WBM at 37 °C or 42 °C (EMCC 1102 and EMCC 1105) for 80 h.

Strains	MRS			WBM		
	Specific Growth Rate (μ)	Doubling Time (t_d)	Time	Specific Growth Rate (μ)	Doubling Time (t_d)	Time
<i>Lc. cremoris</i>	0.021 ^B	33 ^{CB}		0.011 ^D	63.6 ^A	
<i>Lb. bulgaricus</i>	0.039 ^A	17.8 ^D		0.016 ^C	43.3 ^B	
<i>Lb. rhamnosus</i>	0.016 ^C	43.3 ^C		0.018 ^{AB}	38.5 ^C	
<i>Lb. bulgaricus</i> I1	0.013 ^D	53.3 ^B		0.033 ^A	21 ^D	
<i>Lc. cremoris</i> I2	0.014 ^{CD}	49.5 ^{AB}		0.02 ^B	34.7 ^{CD}	
<i>Lc. cremoris</i> I3	0.012 ^D	57.8 ^A		0.018 ^{AB}	38.5 ^C	

A, B, C and D Means with the same letter in the same column are not significantly different.

EPS Productivity

The results of EPS productivity including, utilized sugar, EPS yield, conversion coefficient and biomass dry weight, when EPS producing reference strains isolates grown in MRS broth and Whey Based Medium (WBM) at 37, 42 and 45°C for 64 h, is presented in Figs 2 (a, b, c, d, e & f). The results obviously indicate that the highest EPS production, EPS yield% and conversion coefficient% was achieved at 48 hours of growth in either MRS or WBM at 37 or 42 and 45°C. Data presented in Fig. 2 (a) clear that *Lc. cremoris* DSM 20069 could efficiently utilize glucose and lactose in MRS and WBM, respectively, as sole carbon sources and produce EPS (0.860 and 547 g/l) in MRS and WBM, respectively. Results in Fig. 2 (b) showed that maximum EPS production (0.618 and 0.145 g/l) by *Lb. bulgaricus* EMCC 1105 when grown in MRS and WBM at 42°C for 48 h, respectively. *Lb. rhamnosus* EMCC 1102 grown in MRS and WBM at 45°C for 48 h produced maximum EPS (0.598 and 0.137 g/l) as presented in Fig. 2 (c). Data presented that three isolate strains (*Lactobacillus delbrueckii ssp. bulgaricus* I₁, *Lactococcus lactis ssp. cremoris* I₂ and *Lactobacillus delbrueckii ssp. bulgaricus* I₃) could efficiently utilize glucose and lactose in MRS and WBM, respectively, as sole carbon sources and produce EPS (0.799 and 0.456 g/l) by *Lactobacillus delbrueckii ssp. bulgaricus* I as presented in Fig. 2 (d), EPS production (0.825 0.492 g/l) by *Lactococcus lactis ssp. cremoris* I₂ as

presented in Fig. 2 (e) and EPS production (0.688 and 0.225 g/l) by *Lactobacillus delbrueckii ssp. bulgaricus* I₃ as presented in Fig. 2 (f).

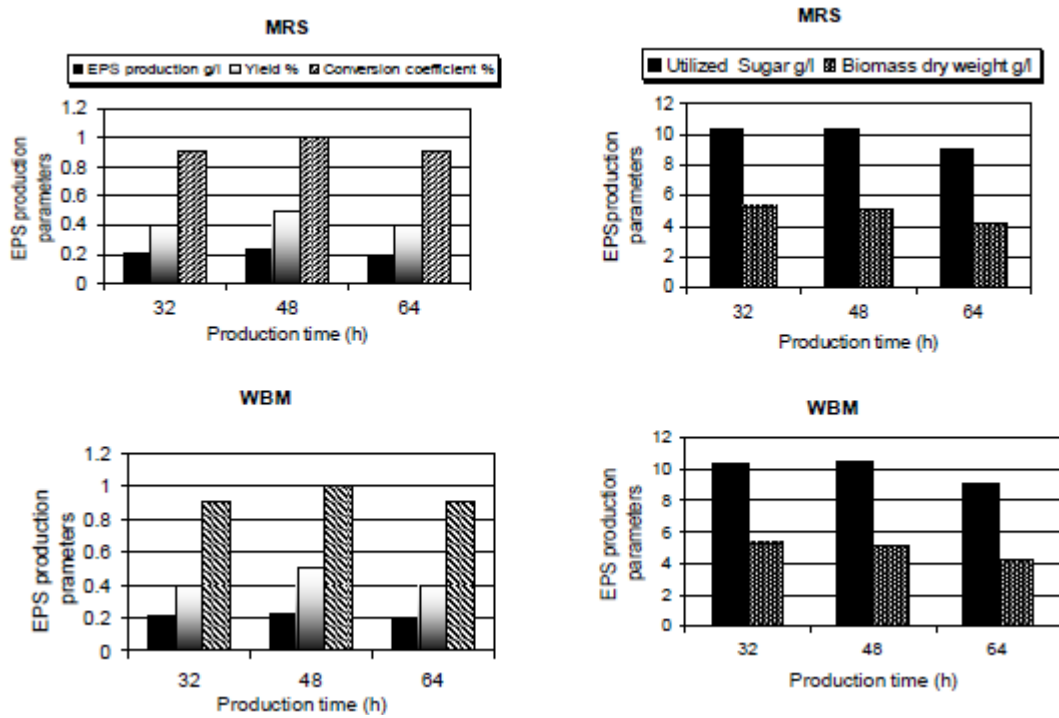


Fig. 2 (a): EPS production parameters of *Lc. cremoris* DSM 20069 grown in MRS and WBM media at 37°C for different periods.

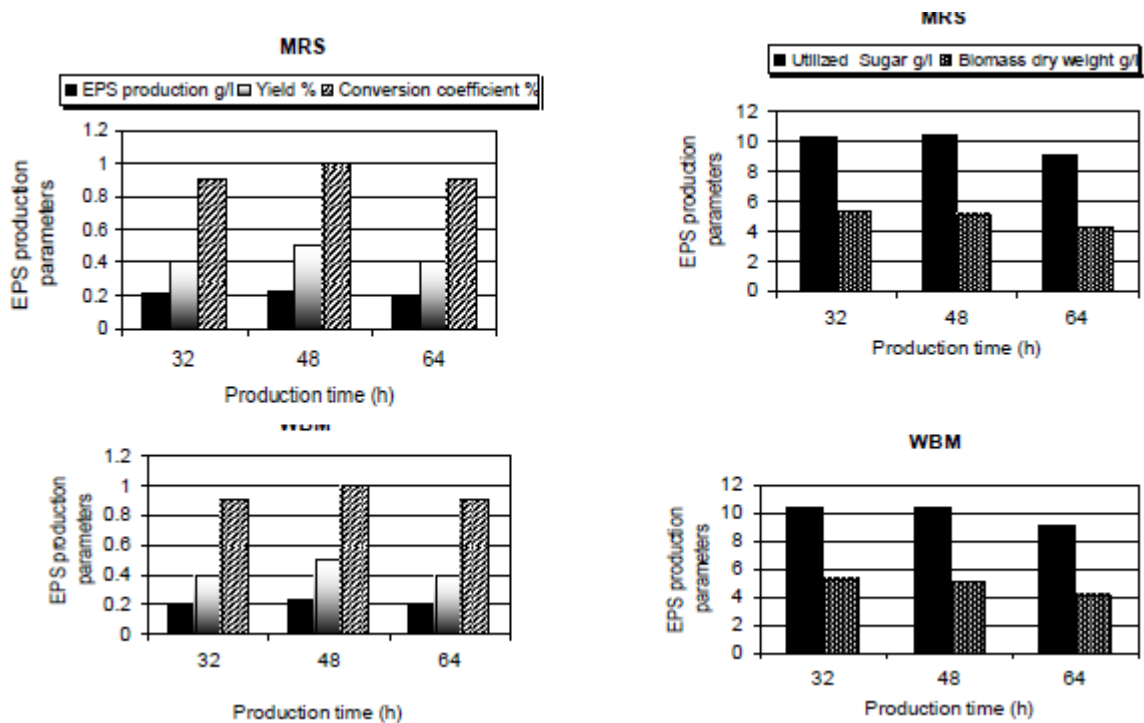


Fig. 2 (b): EPS Production parameters of *Lb. bulgaricus* EMCC 1105 grown in MRS and WBM media at 42°C for different periods.

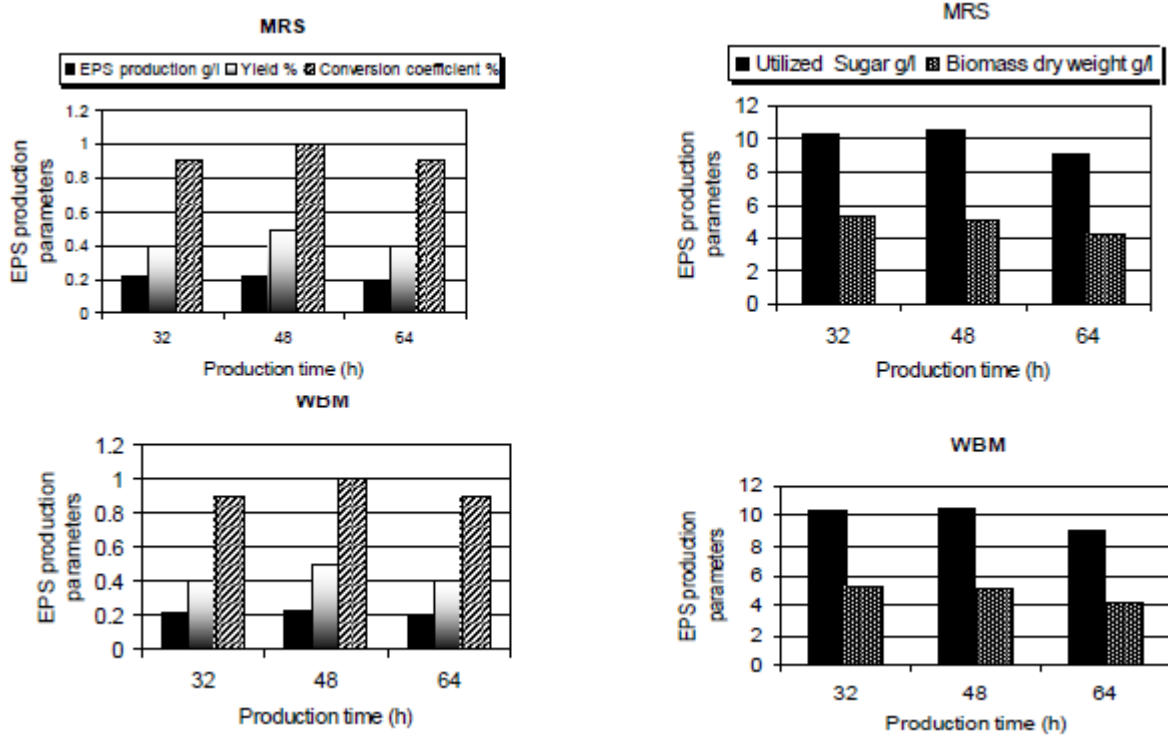


Fig 2 (c): EPS production parameters of *Lb. rhamnosus* EMCC 1102 grown in MRS and WBM media at 45°C for different periods.

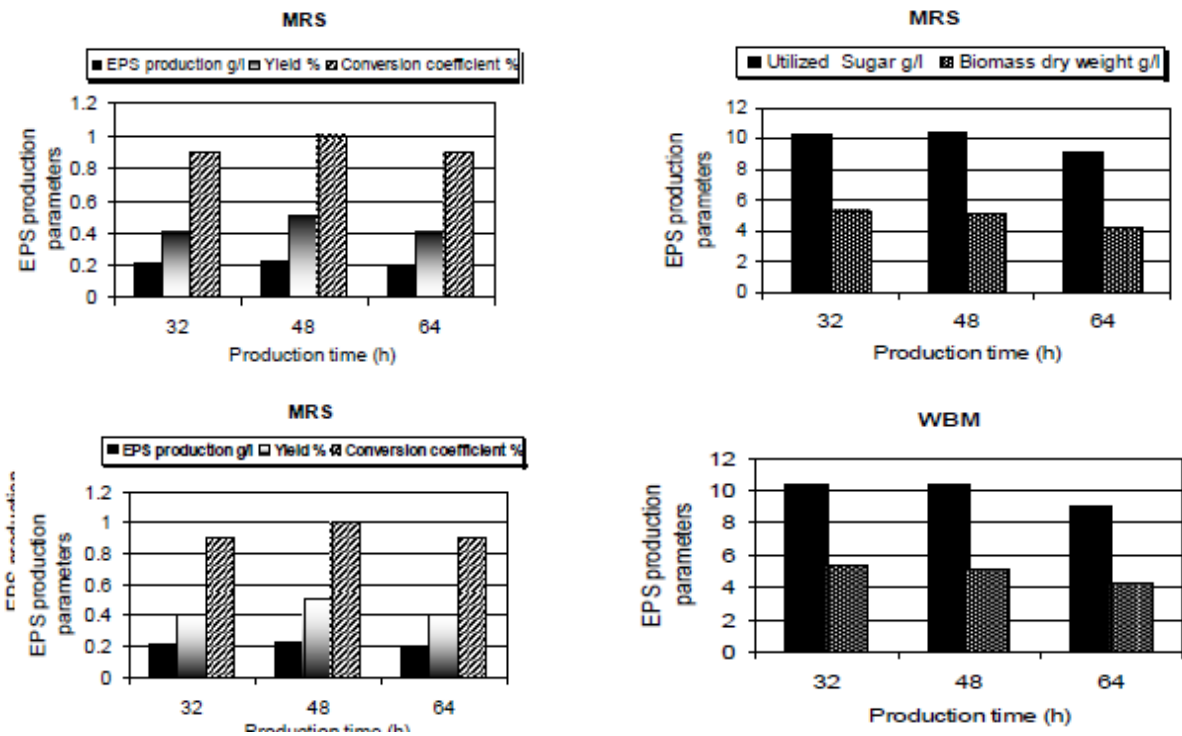


Fig 2 (d): Production parameters of *Lb. bulgaricus* I₁ grown in MRS and WBM media at 42°C for different periods.

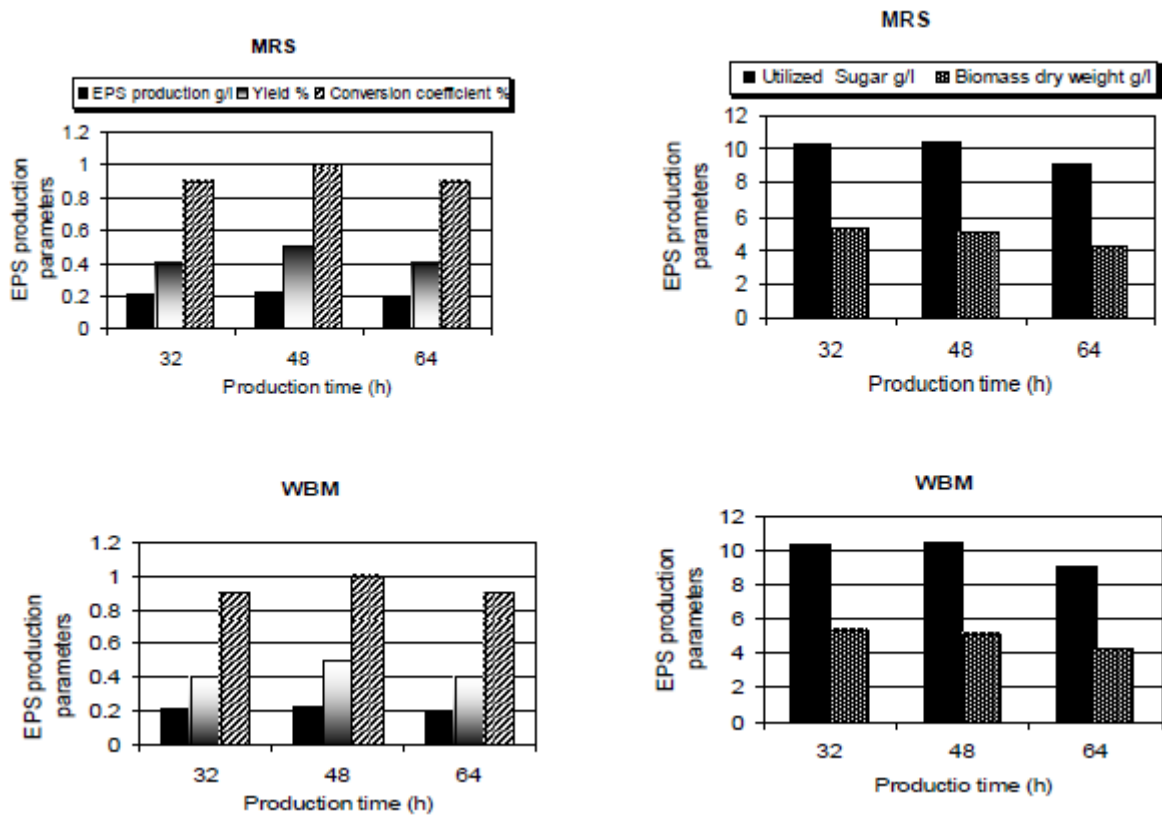


Fig 2(e): EPS production parameters of *Lc. cremoris* I₂ grown in MRS and WBM media at 37°C for different periods.

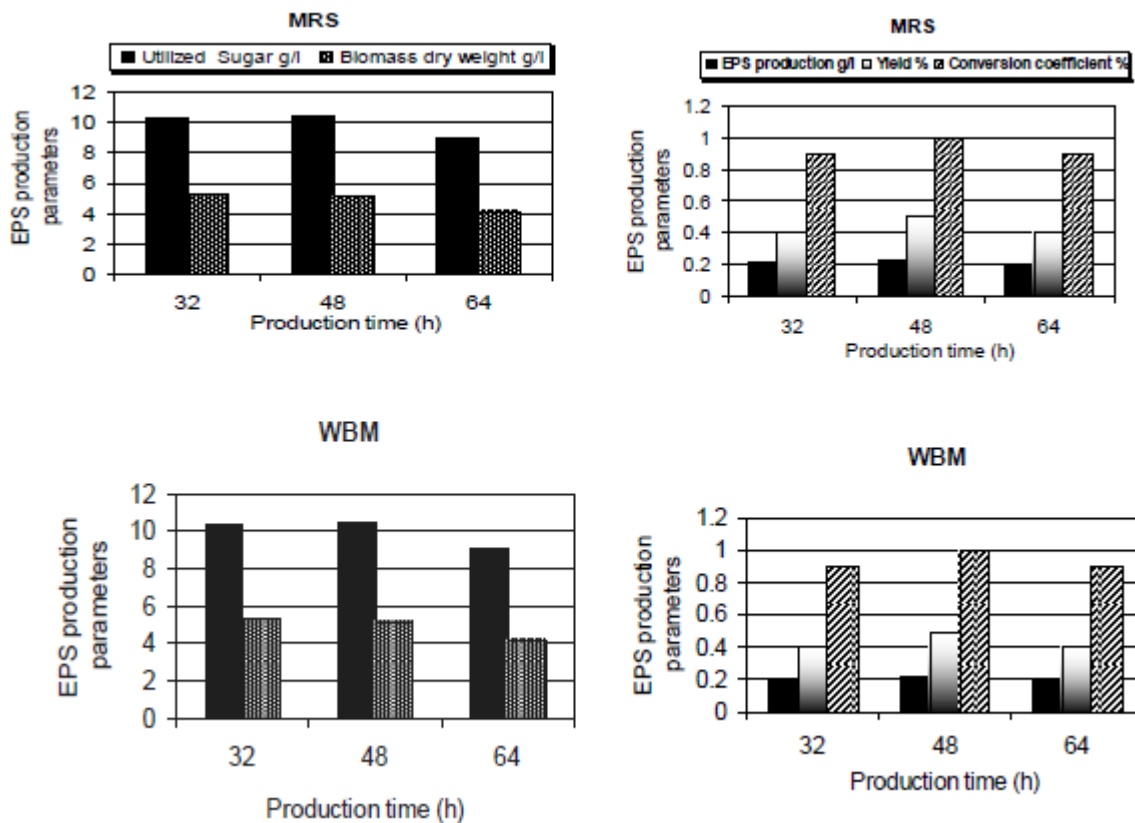


Fig 2 (f): Production parameters of *Lb. bulgaricus* I₃ grown in MRS and WBM media at 42°C for different periods.

Yeast Extract

The data presented in Table (3 and 4) show the effect of supplementing WBM with different yeast Extract levels being 1, 2 and 3 g/l. WBM was supplemented with 100 g/l glucose with initial pH of 6.2, inoculated with 12.5 ml / l of *Lc. cremoris* DSM 20069 and *Lactococcus lactis ssp. cremoris* I₂ incubated at 37°C; and *Lb. bulgaricus* EMCC 1102, *Lb. rhamnosus* EMCC 1105, *Lactobacillus delbrueckii ssp. bulgaricus* I₁ and *Lactobacillus delbrueckii ssp. bulgaricus* I₃ incubated at 42 and 45°C. The fermentation cultures were agitated with speed of 50 rpm.

The results indicated that highest utilized sugar (55 %), EPS production (0.790 g/l), EPS yield (0.79%) and biomass dry weight (6.93 g/l) were achieved by *Lactococcus lactis ssp. cremoris* I₂ in WBM enriched with 3 g/l yeast extract under previously identified optimum conditions for EPS production as presented in Table (3) Regarding the reference strains, at 3 g/L added yeast extract, *Lc. cremoris* DSM 20069 achieved the maximum utilized sugar (64 %), EPS production (0.905 g/l), EPS yield (0.91%) and biomass dry weight (9.15 g/l) as presented in Table (4). Analysis of variance proved that there were significant differences ($P < 0.05$) among treatment examined.

Generally, addition of yeast extract to WBM greatly enhanced the biochemical activity of EPS producing reference and isolated strains. Similar results have been shown by many other investigators⁽³⁰⁻³³⁾.

Table (3): Effect of Yeast Extract Concentration on EPS Production by Isolated LAB Strains inoculated with 12.5 % In WBM (100 g / l glucose, pH 6.2, 50 rpm) and grown at 37 and 42°C For 48 h.

EPS production Parameters	<i>Lb. bulgaricus</i> I ₁			<i>Lc. cremoris</i> I ₂			<i>Lb. bulgaricus</i> I ₃		
	Yeast extract g/L								
	1.0	2.0	3.0	1.0	2.0	3.0	1.0	2.0	3.0
Initial sugar g/l	100								
Final sugar g/l	51.6	49.9	48.9	46.5	46	45	67.3	64.3	63.3
Utilized sugar g/L	48.4	50.1	51.1	53.5	54	55	32.7	35.7	36.7
EPS Production g/l	0.680 ^{C±} 0.635	0.697 ^{B±} 0.635	0.704 ^{A±} 0.635	0.760 ^{C±} 0.519	0.767 ^{B±} 0.635	0.790 ^{A±} 0.635	0.433 ^{C±} 0.635	0.450 ^{B±} 0.635	0.510 ^{A±} 0.635
Biomass dry weight g/l	5.83	5.85	6.00	6.78	6.69	6.93	3.81	3.93	4.1
Yield %	0.86	0.69	0.7	0.76	0.78	0.79	0.43	0.45	0.51
Conversion coefficient%	1.4	1.39	1.37	1.42	1.42	1.44	1.32	1.26	1.39

^a Values are the means ± standard error of at least triplicate measurements.

Means with the same letters are not significantly different.

Table (4): Effect of Yeast Extract Concentration on EPS Production by LAB Strains inoculated with 12.5 % In WBM (100 g / l glucose, pH 6.2, 50 rpm) and grown at 37,42 and 45 °C For 48 h.

EPS production Parameters	<i>Lb. bulgaricus</i> EMCC 1102			<i>Lc. cremoris</i> DSM 20069			<i>Lb. rhamnosus</i> EMCC 1105		
	Yeast extract g/L								
	1.0	2.0	3.0	1.0	2.0	3.0	1.0	2.0	3.0
Initial sugar g/l	100								
Final sugar g/l	70.6	69.8	68.3	39	38.2	36	37	72.1	70.9
Utilized sugar g/l	29.4	30.2	31.7	61	61.8	64	27	27.9	29.1
EPS production g/l	0.350 ^{B±} 0.635	0.366 ^{A±} 0.635	0.413 ^{C±} 0.635	0.882 ^{B±} 0.635	0.893 ^{A±} 0.635	0.905 ^{C±} 0.635	0.277 ^{B±} 0.635	0.298 ^{A±} 0.635	0.340 ^{C±} 0.635
Biomass dry weight g/l	3.2	3.3	3.5	7.71	7.82	8.15	2.70	2.99	3.11
Yield %	0.35	0.37	0.41	0.88	0.89	0.91	0.28	0.3	0.34
Conversion coefficient%	1.2	1.2	1.3	1.4	1.4	1.4	1.0	1.1	1.2

^a Values are the means ± standard error of at least triplicate measurements.

Means with the same letters are not significantly different.

Effect of gamma irradiation on survival and productivity of EPS producing strains.

Radiation is a physical phenomenon in which energy travels through space as a wave motion without the aid of traveling medium. Gamma rays are an ionizing radiation, which have short wavelengths and contain enough energy to ionize the molecules in their paths. The exposure of materials like living cells, foods and medical products to gamma radiation in such a way that precise and specific dose is absorbed is termed "gamma irradiation". Gamma radiation from Co⁶⁰ is the most widely used in practices because of costs and high penetration. On one hand, the depth of penetration of an ionizing radiation depends on the nature of the radiation, the charge of the particles forming it and their energy. On the other hand, it depends on the composition and density of the irradiated substance⁽³⁴⁾. The present experiment was carried out to investigate the effect of gamma radiation on the production of polysaccharides by EPS producing reference strain (*Lc. lactis ssp. cremoris* DSM 20069) and *Lactococcus lactis ssp. cremoris* I₂.

Strains survival

The objective of this experiment was to assess the effect of gamma irradiation on survival and EPS productivity. The most efficient EPS producing reference strain (*Lc. cremoris* DSM 20069) and *Lactococcus lactis ssp. cremoris* I₂ were selected for exposure to gamma irradiation at doses of 0.25, 0.50, 0.75, 1.0, 2.0 and 3 KGy. Both cultures were grown in WBM supplemented with 100 g/l glucose, 3 g/l yeast extract and other identified optimum conditions according to the previous experiments (i.e. initial pH 6.2, inoculum's level 12.5 ml / l, fermentation temperature 37°C, agitation speed 50 rpm for 48h).

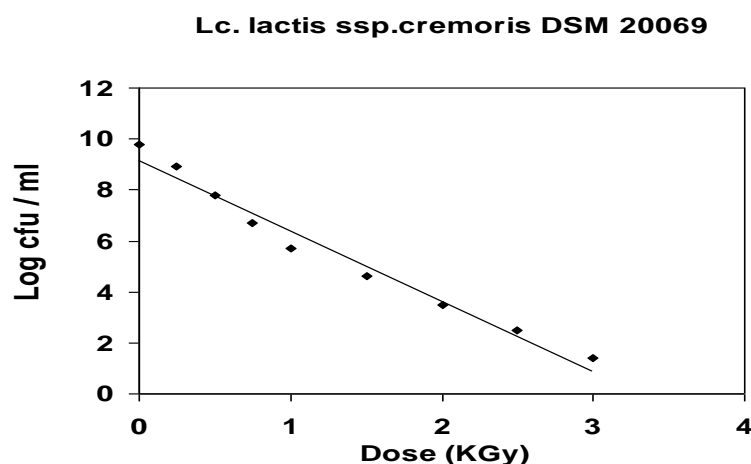
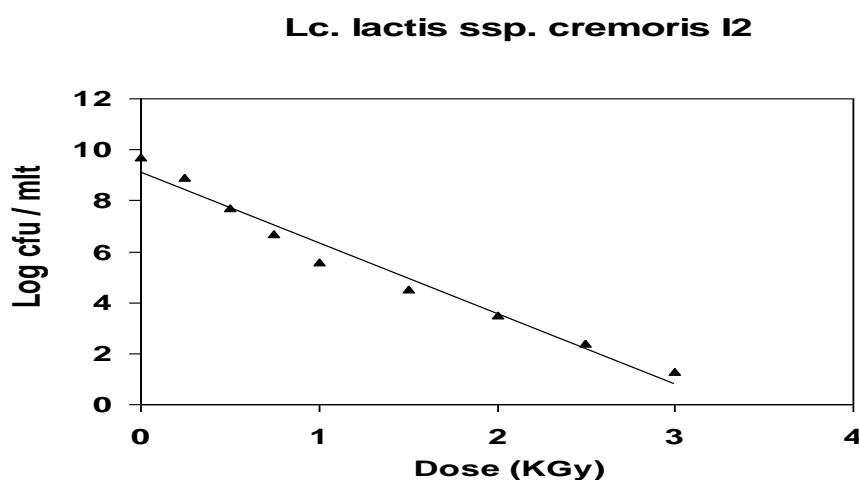


Fig. (3): Effect of different doses of γ -radiation on Survival of *Lc.lactis ssp. cremoris* DSM 20069 strain.

The results of γ - irradiation effect on the reference strain and isolate strain were presented in Fig. (1 and 2). The dose response curves for *Lc. cremoris* DSM 20069 and *Lactococcus lactis ssp. cremoris* I₂ were drawn as illustrated in fig (1 and 2). The strains resistance to gamma irradiation expressed as D10 value (The dose required to reduce the initial population by 90%) was determined from these curves. Fig. (3 & 4) show that the viable count of *Lc. cremoris* DSM 20069 and *Lactococcus lactis ssp. cremoris* I₂ significantly decreased ($P < 0.05$) as irradiation dose increased up to 3.0 KGy. the death of microbial populations from exposure to ionizing radiation is logarithmic in nature. These results also, indicate that gamma irradiation at a dose of 1.5 KGy destroyed 99.9% of both strains. The D₁₀.value (0.348 KGy) for *Lc. cremoris* DSM 20069 and (0.346 KGy) for

Lactococcus lactis ssp. cremoris I₂ indicates the high sensitivity of these strains to Gamma radiation (35).



Fig(4):Effect of Effect of different doses of γ -radiation on Survival of *Lc. lactis ssp. cremoris* I2 strain.

Strains productivity

The results proved that Gamma irradiation of *Lc. cremoris* DSM 20069 and *Lactococcus lactis ssp. cremoris* I₂ at the dose of 0.25 KGY, increased the utilized glucose (64.6, 61.5 %), EPS production (0.976, 0.880 g/l) and EPS yield (0.98, 0.88 %), respectively as presented at Table 3& 4 .However, increasing the radiation does (over 0.25 KGY) caused a significant reduction in the strains productivity. This decrease was proportional with Gamma irradiation does. Several studies recorded that the low doses of gamma radiation may stimulate the microbial growth and metabolic activities (36,37). Meanwhile, high doses of gamma radiation were proved inhibitory for both growth and enzymatic activities of microorganisms (38).

Table (5): Effect of gamma irradiation on EPS productivity by *Lc. cremoris* DSM 20069 grown in WBM at 37°C for 48h, pH 6.2

Fermentation Parameters	Does (KGY)				
	0.00	0.25	0.50	0.75	1.00
Residual sugar (g/L)		35.3	43.6	45.7	58.4
Utilized sugar (g/L)		64.7	56.4	54.3	41.6
EPS production (g/L)		0.976 ^A	0.635 ^B	0.622 ^C	0.418 ^D
		±0.653	±0.653	±0.577	±0.653
Biomass dry weight (g/L)		8.81	5.41	5.27	3.66
Yield%		0.98	0.64	0.62	0.42
Conversion coefficient%		1.5	1.7	1.2	1.0

- Initial sugar (100 g/L).
- A, B, C and D Means with the same letter in the same row are not significantly different.
- Values are the means \pm standard error of at least triplicate measurements.

Initial sugar (100 g/L) . A, B, C and D Means with the same letter in the same row are not significantly different. Values are the means \pm standard error of at least triplicate measurements Table (5&6). This improvement in fermentation characteristics could be attributed to the stimulatory effect of gamma irradiation for certain enzyme systems in EPS producing strains or from the effect of γ radiation on gene responsible for production of EPS, The exposure of cells to ionizing radiation sets off a chain of reactions giving rise to chemical and then to metabolic or physiological changes. The irradiation presents an additional stress to the cell, which tend to disturb their organization⁽³⁹⁾. From these results, it is concluded that, the use of γ radiation as a mutagenic agent may be useful for production of mutant strains from *Lc. cremoris* DSM 20069 and *Lactococcus lactis ssp. cremoris* I₂ capable of producing higher in WBM at 37°C for 48h, pH 6.2. quantities of EPS. Statistical analysis proved that gamma irradiation of the two mentioned strains at dose of (0.25 KGy) significantly enhanced ($p < 0.05$) glucose utilization, EPS production and EPS yield when these strains grown in WBM at optimum conditions for EPS production.

Table (6): Effect of gamma irradiation on EPS productivity by *Lc. cremoris* I₂ grown WBM

Fermentation Parameters	Dose (KGY)			
	0.25	0.50	0.75	1.00
Residual sugar (g/L)	38.5	42.7	47.2	56
Utilized sugar (g/L)	61.5	57.3	52.8	44
EPS production (g/L)	0.880 ^A	0.620 ^B	0.537 ^C	0.380 ^D
	± 0.653	± 0.653	± 0.577	± 0.577
Biomass dry weight (g/L)	7.88	5.31	4.29	3.16
Yield%	0.88	0.62	0.54	0.38
Conversion coefficient%	1.4	1.1	1.0	0.87

Surfactants

In this experiment, three surface-active agents, (tween 40, tween 80 and triton x-100) were added, in different concentrations (0.25, 0.5 and 1.0 ml / l) to WBM with optimal condition identified through the previous experiments. The aim was to improve the productivity of EPS by irradiated strains *Lc. cremoris* DSM20069 and *Lactococcus lactis ssp. cremoris* I₂ exposed to 0.25 KGy. The results are presented in table (7 and 8). The highest utilized sugar (65.4 %), EPS production (0.985 g/l), EPS yield (1%) and biomass dry weight (9.02 g/l) were achieved by irradiated (0.25 KGy) *Lc. cremoris* DSM 20069 grown in WBM treated with 0.5 g/l triton x-100 as presented in Table (5). regarding the *Lactococcus lactis ssp. cremoris* I₂, the highest utilized sugar (62.5 %), EPS production (0.887 g/l), EPS yield (0.89%) and biomass dry weight (7.93 g/l) were achieved by irradiated (0.25 KGy) *Lactococcus lactis ssp. cremoris* I₂ grown in WBM treated with 0.5 g/L triton x-100 as presented in Table (6). It could be observed that gamma irradiation (0.25 KGy) of *Lc. Cremoris* DSM 20069, *Lactococcus lactis ssp. cremoris* I₂ significantly enhanced EPS production, and EPS yield% when grown in WBM treated with 0.5g/l triton x-100 under optimum fermentation conditions. As a conclusion, presence of 0.5g/L triton x-100 in the fermentation medium obviously increased the EPS production. These results coincide with those obtained, who reported that addition of some surface active agents such as tween 40, tween 80 and triton x-100, to fermentation media improved final

polysaccharide concentration, viscosity profiles and the rheological quality of the polymer compared with the control (without surfactants)⁽⁴⁰⁾.

Table (7): Effect of some surfactants on EPS Production by irradiated (0.25 KGy) *Lc. cremoris* DSM 20069 grown in WBM (100 g / l glucose, pH 6.2, 50 rpm) at 37°C For 48 h.

EPS production Parameters	Surfactants								
	Tween 40			Tween 80			Triton x-100		
	Surfactants Level ml/l								
	0.1	0.25	0.5	0.1	0.25	0.5	0.1	0.25	0.5
Initial sugar g/l	100								
Final sugar g/l	37.2	36.5	35.5	37.5	36.5	36.3	36.9	35.8	34.6
Utilized sugar g/l	62.8	63.5	64.5	62.5	63.5	63.7	63.1	64.2	65.4
EPS production g/l	0.933 ^{C±} 0.577	0.946 ^{B±} 0.577	0.965 ^{A±} 0.577	0.927 ^C 0.577	0.946 ^B 0.577	0.953 ^A 0.577	0.938 ^C 0.577	0.963 ^{B±} 0.577	0.985 ^A 0.577
Biomass dry weight g/l	8.21	8.36	8.57	8.19	8.36	8.42	8.21	8.55	9.02
Yield %	0.93	0.95	0.97	0.93	0.95	0.95	0.94	0.96	1
Conversion coefficient%	1.4	1.5	1.5	1.4	1.5	1.5	1.5	1.5	1.6

^a Values are the means ± standard error of at least triplicate measurements.

Means with the same letters are not significantly different.

Table (8): Effect of some surfactants on EPS Production by irradiated (0.25 KGy) *Lc. cremoris* I2 grown in WBM (100 g / l glucose, pH 6.2, 50 rpm) at 37°C For 48 h.

EPS production parameters	Surfactants								
	Tween 40			Tween 80			Triton x-100		
	Surfactants Level ml/l								
	0.1	0.25	0.5	0.1	0.25	0.5	0.1	0.25	0.5
Initial sugar g/l	100								
Final sugar g/l	40.7	40.2	38.9	40.7	40.5	40.1	40.3	39	37.5
Utilized sugar g/l	59.3	59.8	61.1	59.3	59.5	59.9	59.7	61	62.5
EPS production g/l	0.822 ^{C±} 0.577	0.875 ^{B±} 0.577	0.878 ^{A±} 0.577	0.821 ^{C±} 0.577	0.837 ^{B±} 0.577	0.865 ^{A±} 0.577	0.840 ^{C±} 0.577	0.876 ^{B±} 0.577	0.887 ^{A±} 0.577
Biomass dry weight g/l	5.8	7.61	7.65	7.00	7.48	7.60	7.55	7.70	7.93
Yield %	0.82	0.88	0.88	0.82	0.84	0.87	0.84	0.89	0.89
Conversion coefficient%	1.4	1.5	1.5	1.4	1.4	1.5	1.4	1.5	1.6

Values are the means \pm standard error of at least triplicate measurements ⁽⁴¹⁾. stated that increasing the cell membrane permeability, obviously enhanced the glutamate transport system. Also saturated fatty acid (C: 16-C: 18) and their ester derivatives (tweens) repress acetyl-CoA carboxylase. Adding such surfactants at logarithmic growth phase, would led to synthesize all membranes with lower phospholipids content, but higher in saturated fatty acid content of lipids. Such changes lead to decrease the membrane lipids by \sim 50%. This would greatly enhance the permeability of the cell membrane, which obviously increases the EPS amount released into the growth medium.

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المؤتمر الدولي الثاني للعلوم الإشعاعية وتطبيقاتها

مستخلص الخميرة

في هذه التجربة تم تدعيم بيئة الشرش بواسطة مستخلص الخميرة كمصدر لعوامل النمو عند تركيز 1 ، 2 ، 3 جم/لتر والنتائج 0 و قد أشارت النتائج إلى ما يلي:

تم الحصول على أكبر إنتاج للسكريدات العديدة (0.905 جم/لتر) بواسطة سلالة *Lc. lactis ssp. cremoris* DSM 20069 و تبعه إنتاج مقداره 0.790 جم/ لتر تم الحصول عليه بواسطة *Lc. lactis ssp. cremoris* I2

تأثير أشعة جاما على إنتاج السكريدات العديدة بواسطة بكتيريا حمض اللاكتيك

تم في هذه التجربة تعريض أعلى سلالتين إنتاجية للسكريدات العديدة وهما *Lc. lactis ssp. cremoris* DSM 20069 و *Lc. lactis ssp. cremoris* I2 للجرعات مختلفة من أشعة جاما مقدارها (0.0، 0.25، 0.5، 0.75، 1.0، 1.5، 2.0، 2.5، 3.0، kGy) وذلك باستخدام الجرعة الإشعاعية 5.38 kGy/h

وقد تم رسم منحني الاستجابة للجرعات الإشعاعية و ذلك بتوقيع عدد الخلايا المتبقية مقابل قيمة الجرعة الإشعاعية ، فضلا عن دراسة تأثير أشعة جاما على خصائص السلالتين. و قد أشارت النتائج الى أن:

أشعة جاما عند الجرعة 1.5 KGy قتلت 90% من عدد الخلايا للسلالتين مما يدل على حساسيتهما لأشعة جاما. تعرض السلالتان *Lc. lactis ssp. cremoris* DSM 20069 و *Lc. lactis ssp. cremoris* I2 لأشعة جاما عند الجرعة 0.25 KGy أدى لزيادة السكر المستهلك (64.7 ، 61.5 جم/لتر) و إنتاج السكريدات العديدة (0.976 ، 0.880 جم/لتر) و محصول السكريدات العديدة (0.88، 0.98 %) لكنتا السلالتين على التوالي.

تأثير المواد ذات التوتر السطحي

في هذه التجربة تم معاملة بيئة الشرش بواسطة أنواع مختلفة من المواد ذات التوتر السطحي مثل Tween 80, Triton x-100 و 40 بتركيزات 0.1، 0.25، 0.5 مل/لتر و كانت النتائج كالآتي:

تم الحصول على أعلى قيم للسكر المستهلك (65.4 جم/لتر)، و إنتاج السكريدات العديدة (0.985 جم/لتر) ، و محصول السكريدات العديدة (1%) و الوزن الجاف للخلايا (9.02 جم/لتر) بواسطة *Lc. lactis ssp. cremoris* DSM 20069 المشبعة بجرعة 0.25 و كيلو جرای و المنماه في بيئة الشرش المعالجة بـ 5 مل/لتر Tritonx-100

وبالمثل فإن السلالة *Lc. lactis ssp. cremoris* I2 المشبعة بنفس الجرعة و المنماه في بيئة الشرش المعالجة بـ 5 مل/لتر Tritonx-100 قد أعطت أعلى قيمة للسكر المستهلك (62.5 جم/لتر) و إنتاج السكريدات العديدة (0.887 جم/لتر) و الوزن الجاف للخلايا (7.93 جم/لتر). و خلاصة نتائج هذه الدراسة أن الشرش بيئة إقتصادية قيمة لإنتاج السكريدات