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Exopolysaccharides Produced by Pure Culture of *Lactobacillus, Lactococcus* and Yeast Isolated from Kefir Grain by Microtiter Plate Assay: Optimization and Comparison

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Abstract: A fast, repeatable and reliable method for kefiran production by *Lactobacillus delbrueckii subsp bulgaricus, Lactobacillus delbrueckii subsp lactis, Entrococcus facium, Lactococcus lactis,* acetic acid bacteria and yeast was established, in which the effects of heat, carbohydrate and nitrogen sources on cell growth, exopolysaccharide formation and substrate assimilation were considered. This method was used for optimization and comparison of EPS production in microorganism isolated from Kefir. *Lactobacillus delbrueckii subsp bulgaricus,* (10^8 CFU/ml) *Lactobacillus delbrueckii subsp lactis* (10^5CFU/ml), *Entrococcus facium* (10^8-10^9 CFU/ml), *Lactococcus lactis*, (10^8-10^9 CFU/ml) Acetic acid bacteria (10^4-10^5 CFU/ml) and yeast (10^6 CFU/ml) were isolated and enumerated from kefir. For optimizing EPS production from these isolates, pure cultures were used in microplate and EPS were assayed as biofilm formation. *Lactobacillus delbreuckii subsp bulgaricus* was the best strain for EPS production and a chemically defined medium with 6% lactose, 2% pepton and yeast extract and incubation at 37°C was the best culture condition for EPS production, however the isolated Cocci had maximum EPS on glucose and galactose, incubated at 37°C and no EPS produced from isolated yeast and acetic acid bacteria. It was concluded that the method used was a fast, cheap and accurate assay for evaluating the effect of different conditions on Exopolysaccharide production in pure culture of microorganisms.

Key words: Exopolysaccharide · Kefiran · *Lactobacillus* · *Streptococcus* · Yeast · Microtiter

INTRODUCTION

Exopolysaccharide produced by lactic acid bacteria have generated attention among researchers for the last few years. They contribute to the specific rheology and texture of fermented milk products and when they are added to food they act as an emulsifiers, thickeners, stabilizers and gelling agents [1]. Exopolysaccharide production is an important feature of lactic acid bacteria in forming starters for fermented milk product. In addition exopolysaccharides have benefits in human health and have an important role against gastrointestinal diseases as they act as a non- digestible food fraction [2,3] and they also have antitumor activities [4]. Kefir is fermented milk which is prepared by inoculation of milk with kefir grain as starter. These grains consisted of slimy material in which yeast and bacterial cells are firmly embedded. The microflora of kefir grain is principally Lactic acid bacteria, yeast and acetic acid bacteria. At least one quarter of the dry mater of kefir grain consist of a capsular polysaccharide denominated as kefiran [5]. Although *Lactobacillus kefir* is responsible for kefiran production Kandler and Kunath [6] concluded that *Lactobacillus kefir* was not a kefiran producer. However, according to other reports the principle producer of kefiran polymer is *lactobacillus kefiranofaciens* and other unidentified species of lactobacillus [7,8]. Thus it remains unknown which microorganism is responsible for kefiran production in kefir grain. The potential health properties of kefiran have prompted several groups to develop media and growing conditions that optimize kefiran production. Media based on lactic acid and whey has been found to be optimum for kefiran production [9]. A batch procedure using a modified MRS media was reported by Micheli *et al.* [10]. Mitseu *et al.* [8] combined the bacterium, *lactobacillus kefiranofaciens*, with the yeast

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Torulaspora delbrueckii. When these two organisms were grown in 50 l bioreactor in a fed-batch protocol, a yield of 3740 mg/l was obtaind over a 7 day period. Also Tada *et al.* [11] used a fed batch system to enhance the production of kefiran with a Coculture of *Lactobacillus kefiranofaciens* with *Saccharomyces cerevisiae*. Different exopolysaccharide (EPS) assays have been developed and used by many researchers over the last two decades. All these methods involve a combination of techniques to isolate, purify and quantify the EPS in a culture medium. Some of the techniques used to isolate and or purify EPS include size-exclusion chromatography, anion exchange chromatography, ultra-filtration, dia-filtration, centrifugation, dialysis, ultrasonication and lyophilisation. Common techniques used for precipitating EPS include ethanol, acetone, propanol, isopropanol, cetyltrimethylammonium bromide (CTAB) and 3,5,6-triphenyl- 2,3,5,6-tetraaza bicycle-1-hexene (commercially known as Nitron). In culture media containing proteins, trichloroacetic acid (TCA) and enzyme treatments have been commonly employed to precipitate and hydrolyse the proteins, respectively. The quantification of EPS subsequent to isolation and purification commonly employs the phenol-sulphuric acid method [12]. For complex media containing milk, the EPS values are inaccurate due to the non-EPS components present in the media which often interfere with the assay [12]. Culture medium containing yeast extract could also interfere with EPS assay due to the presence of mannans. Complex media are commonly used in studies examining exopolysaccharide production by *Lactobacillus delbrueckii* ssp. *bulgaricus*. However, quantification of exopolysaccharide in complex medium can be complicated by interference due to carbohydrate polymers contained in media components. A study was undertaken to identify components of MRS, a common medium for cultivation of *L. delbrueckii* ssp. *bulgaricus* that interfere with exopolysaccharide quantification by kimmel *et al.* [13] Phenol-sulfuric acid determinations were conducted on uninoculated MRS broth with and without yeast extract, beef extract and peptone. These three ingredients accounted for 94% of the total background exopolysaccharide-equivalent in MRS broth. However in both milk and non milk based media the methods used for measuring the amount of exo polysaccharides in all cases are time consuming and for evaluating the effect of different conditions on EPS production a high amount of medium and component has to be consumed.

The aim of this study was to isolate the best EPS producer strain from kefir grain and optimization of EPS production in pure cultures by using a fast and reliable method micro titer plate assay in purpose to use these conditions for EPS enhancement in kefir.

MATERIALS AND METHODS

Kefir grains were obtained from a private household, washed with distillated water and inoculated in cow milk (2% fat) and after 24 hours the grain were separated from the milk by filtering them through a sieve and washed for later use while the grains were not being used they were preserved in milk at 4°C.

Microbiological Analysis: Peptone water at a concentration of 0.1% was used to prepare the dilutions for the microbiological analysis. Surface seeding and pour plate were used in all cases. *Lactobacillus* counts were performed on MRS medium obtained from Merck Company, at an incubation of 37°C under anaerobic conditions (5% CO_2) for 3 days. Cocci counts were carried out on M_{17} medium obtained from Merk at an incubation temperature of 37°C under aerobic conditions for 2 days. Yeasts were grown on PDA medium obtained from Merk Company at 25°C under aerobic conditions. Acetic acid bacteria were grown on GYC medium containing 20 g/l glucose, 10 g/l yeast extract and 30 g/l $CaCO_3$. Stock cultures were stored at 75°C in 50% glycerol.

Identification of Isolated Strains: For biochemical profiles of lactic acid bacteria basal MRS media without meat extract and glucose were used and cultivation of carbohydrates were analyzed. Growth in different temperature 10, 45°C and growth in 6% NaCl were investigated in MRS medium.

Microplate Titer Assay: An overnight culture of each strain was prepared in the optimum conditions mentioned above Biofilm production assays were performed with a chemically defined media. After vortexing the overnights, 50µl volumes were transferred into eight PVC microtiter plate. The basal medium (MRS without glucose) was substituted with 8 sugar (glucose, galactose, sucrose, fructose, lactose, ribose, mannose.) at different concentration (1, 2, 3, 4, 5, 6, 8 and 10%). 200 microliter of these media were transferred to each microplate. Another medium was prepared with the same basal MRS without nitrogen source substituted with peptone casein, meat extract and yeast extract at different concentration. after 24 hours incubation at different temperatures (25, 30 , 37, 45°C), with CO_2 and without CO_2, medium was removed

from wells and microtiter plate wells were washed two times with sterile distilled water to remove loosely associated bacteria. Each well was stained with 200 microliter ethanol 95% and ethanol was removed after 15 min and the plates were air dried for 45 min and each well was stained with 150 µl of 1% crystal violet solution for 5 min. After staining, plates were washed with sterile distilled water. At this point, biofilms were visible as purple rings formed on the side of each well. The wells were left to dry and after drying each well was stained with acetic acid 33% and the absorbance was read in an ILISA reader.

Statistical Analysis: Statistical analyses were carried out with SAS software and T- test.

RESULTS AND DISCUSSION

The microbial population found in kefir grain has been used as an example of a symbiotic community; this symbiotic nature make identification and study of the constituent microorganism in grain difficult. In this research several media has been used for isolation and identification of bacteria in kefir with or without CO_2 on MRS, M_{17} and PDA. As it is shown in Table 1 the number of *Lactobacillus,* Coccus, yeast and Acetic acid bacteria in kefir were (10^7-10^8), (10^8-10^9), (10^6-10^7) and (10^5) respectively which are in accordance with others [14-15]. Although cultivation conditions can affect the population pattern, Garrote *et al.* [16] showed that lactic acid bacteria are more numerous than yeast and acetic acid bacteria.

As it is shown in Table 2 the microorganism isolated and identified with biochemical test according to Bergey's manual to systematic bacteriology were recognized as, *Lactobacillus delbrueckii* ssp *lactis, Lactobacillus delbrueckii* ssp *bulgaricus, Entrococcus facium, Lactococcus lactis.* Yeasts were also isolated from kefir and they were only recognized by morphological characteristics. Microorganisms isolated from GYC were recognized as *Acetobacter* according to Bergery. Most reports on the microbiology of kefir indicated the existence of *Lactococcus, Lactobacillus, Streptococcus, Entrococcus, Leuconostoc,* yeast and Acetic acid bacteria in kefir [4], however other bacteria such as *Bacillus, Micrococcus* and *Escherichia* are also found in kefir. The yeast found in kefir grains provides an environment for growth of kefir bacteria and provides essential growth nutrients such as amino acid and vitamins. The effect of different strains isolated from Kefir grains (*Lactobacillus delbrueckii spp bulgaricus, Lactobacillus delbrueckii spp lactis, Lactococcus lactis, Enterococcus facium, Acetobacter* and yeast) on EPS production were studied as biofilm formation in microplates. Also the effect of incubation temperature, different carbohydrate and concentration of carbohydrate on EPS production were measured by microtiter plate assay. The results for biofilm production in different strains are shown in Table 3. As it is shown *Lactobacillus delbrueckii spp bulgaricus* was the best EPS producer in media with lactose as a source of carbon. The results were statistically significant at $p<5\%$. This strain produced more EPS in all different carbohydrates compared to the other isolated strains. Statistically this strain shows a significant difference in EPS production in presence of all different carbohydrates, except *Lactococcus lactis* in presence of galactose, which also has a compatible EPS production. Moreover *Entercoccus facium* in presence of glucose produced a considerable amount of EPS. Production of kefiran from hemofermentive *Lactobacillus Kefiranofaciens* SP.KPB.167B in MRSL and MRS media were studied by Yokoi *et al.* [7]. They Could produce 2.04 gram kefiran on a media with 10% lactose, 5mM $CaCl_2$, 30°C and pH=5. The production of EPS also were studied by Kimmel *et al.* Petry *et al.* and Aslim *et al.* [17-19] in *Lactobacillus delbrueckii spp bulgaricus* as an appropriate strain in EPS production. The results indicated that the most effective factors to produce EPS are pH, oxygen, temperature and carbon sources.

Ferengova *et al* in 2002 isolated *Lactobacillus delbrueckii* spp *bulgaricus, Lactobacillus helveticus, Lactobacillus brevis* and *Streptococcus lactis* from kefir and obtained the highest amount of EPS (400 mg/l EPS) from *Lactobacillus delbrueckii* spp *bulgaricus.* They found out that the only EPS produced by this strain is a polymer with 1:1 ratio of glucose to galactose. Therefore the produced EPS was identified as kefiran. The same result was found in this work and *Lactobacillus delbrueckii* spp *bulgaricus* produced the highest amount of EPS that it must be kefiran. In addition it was found that *Lactococcus* and *Entrococcus* isolated from Kefir produce EPS as well and form biofilm in microplate. The same results also have been seen in other researches [20]. The high amount of EPS from *Entococcus facium* and *Lactococcus lactis* were obtained in media with glucose and galactose as the carbon sources respectively. As it is shown in Table 3 the isolated yeast and *Acetobacter* did not have any EPS and the results were confirmed by loop test too (Table 4). Biofilm formation has been studied by Djordjevic *et al.* for *Listeria monocytogenes*, Rice *et al.* [21] for serratia marcescenc. They found that biofilm

Table 1: Enumeration of microbial population in kefir (CFU/ml)

Enumeration	Method: Pour plate	Method: Surface seeding
Total population of *Lactobacillus* (MRS)	$1*10^8$ - $7*10^7$	$5*10^7$-$3*10^7$
Total population of Cocci (M_{17})	$1*10^9$-$2.4*10^8$	$7*10^7$-$1.5*10^8$
Total population of yeast (PDA)	$7*10^6$-$1.1*10^7$	$1*10^7$-$1*10^7$
Total population of *Acetobacter* (GYC)	$1*10^5$-$4*10^5$	$1.6*10^5$- $2*10^5$

Table 2: Identification of isolated bacteria from kefir by biochemical test

Biochemical test	*Lactobacillus delbrueckii* spp *lactis* isolate Ia	*Lactobacillus delbrueckii* spp *lactis* isolate Ib	*Lactobacillus delbruekcii* spp *lactis*	*Lactobacillus delbrueckii* spp lactis (Bergey) isolate Ic	*Lactobacillus debrueckii* spp *bulgaricus* isolate II	*Lactobacillus debrueckii* spp *bulgaricus* Bergey	*Entrococcus facium* A	*Entrococcus facium* B	*Entrococcus facium* C	*Entrococcus facium* (Bergey)	*Lactococcus lactis* E	*Lactococcus lactis* D	*Lactococcus lactis* (Bergey)
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+
Gatalase	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	-	-	-	-	+	+	+
Sucrose	+	+	+	+	-	-	-	-	-	-	+	+	+
Surbitol	-	-	-	-	-								
-				ND			ND						
Rammnose	-	-	-	-	-	-				ND			ND
Lactose	+	+	+	+	+	+	+	+	+	+			ND
Ribose	-	-	-	-	-	-				ND	+	+	+
Mannitole	-	-	-	-	-	-	-	-	-	-			ND
Maltose	+	+	+	+	+	-				ND	+	+	+
Mannose	+	+	+	+	-	-				ND	+	+	+
Gluconate	-	-	-	-	-	-				ND			ND
Galactose	+	+	+	D	-	-				ND	+	+	+
Melezitose	-	-	-	-	-	-	-	-	-	-			ND
Fructose	+	+	+	+	+	+	-	-	-	-	+	+	+
Tryhalsoe	+	+	+	+	-	-				ND	+	+	+
Raffinose	-	-	-	-	-	-	-	-	-	-			ND
Melebiose	-	-	-	-	-	-	+	+	+	+			ND
Spore formation	-	-	-	-	-	-	-	-	-	-	-	-	-
Gas from glucose	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 15				-	-	-	-	-	-	-	+	+	+
Growth at 45				+	+	+	+	+	+	+	-	-	-
Growth at 6.5% Nacl				ND		ND	+	+	+	+	-	-	-
Growth at pH 9.6				ND		ND	+	+	+	+	-	-	-

+: 90% or more strains are positive

-: 90% or more strains are negative

D: 11-80% strains are positive

ND: Not determined

Table 3: biofilm assay as indicator of EPS production in pure culture of *Lactobacillus*, Cocci, yeast and *Acetobacter* in 1% (w/v) carbohydrates incubated for 24 hr at 37 °C

Carbon source	*Entrococcus facium* A	*Entrococcus facium* B	*Entrococcus facium* C	*Lactococcus lactis* D	*Lactococcus lactis* E	*Lacto bacillus delbrueckii* spp *lactis* isolate Ic	*Lacto bacillus delbrueckii* spp *lactis* isolate II	*Lacto bacillus delbrueckii* spp *lactis* isolate Ia	*Lactobacillus debrueckii* spp *bulgaricus*	*acetobacter*	yeast
Glucose	0.525 ^{a}	1.204 ^{b}	1.159 ^{b}	0.398 ^{c}	0.721 ^{a}	0.351 ^{c}	0.420 ^{ac}	0.360 ^{c}	1.901 ^{d}	0.017 ^{e}	0.019 ^{e}
Galactose	0.040 ^{a}	0.033 ^{a}	0.384 ^{b}	0.443 ^{b}	1.396 ^{c}	0.012 ^{d}	0.391 ^{b}	0.368 ^{b}	1.416 ^{c}	0.024 ^{ad}	0 ^{e}
Sucrose	0.020 ^{a}	0.220 ^{b}	0.027 ^{a}	0.011 ^{c}	0.032 ^{a}	0.038 ^{a}	0.011^{c}	0.028 ^{a}	0.943 ^{d}	0.010 ^{c}	0.004 ^{c}
Fructose	0.084 ^{a}	0.381 ^{b}	0.301 ^{b}	0.357 ^{b}	0.188 ^{ac}	0.209 ^{c}	0.240 ^{cb}	0.237 ^{cb}	0.474 ^{d}	0.011 ^{e}	0.012 ^{d}
Lactose	0.010 ^{a}	0.188 ^{b}	0.065 ^{ab}	0.110 ^{a}	0.018 ^{a}	0.031 ^{a}	0.036 ^{a}	0.034 ^{a}	2.376 ^{c}	0.007 ^{a}	0.029 ^{a}
Ribose	0.108 ^{a}	0.471 ^{b}	0.340 ^{b}	0.038 ^{c}	0.016 ^{c}	0.050 ^{c}	0.236 ^{ab}	0.044 ^{c}	1 ^{d}	0.008 ^{c}	0.013 ^{c}
Mannose	0.036 ^{a}	0.228 ^{bc}	0.012 ^{a}	0.390 ^{c}	0.191 ^{b}	0.029 ^{a}	0.031 ^{a}	0.030 ^{a}	0.595 ^{d}	0 ^{e}	0.009 ^{e}

Means in columns with the same letter are not significantly different at P=0.05

Table 4: EPS production by loop test (mm)

Media Isolates	MRS	MRS+1% glucose	MRS+ 2% glucose
Entrococcus facium A	1	2-3	4-5
Entrococcus facium B	1	2-3	6-7
Entrococcus facium C	1	4-5	4-5
Lactococcus lactis D	0.5	2-3	2-3
Lactococcus lactis E1	1-2	2-3	
Lactobacillus delbrueckii spp *lactis* Ia	1	2-3	1-2
Lactobacillus delbrueckii spp *lactis* Ib	1	1-2	1
Lactobacillus delbrueckii spp *lactis* Ic	0.5	1	-
Lactobacillus delbrueckii spp *bulgaricus isolate II*	2-3	4	7-8
Acetobacter	0.5	-	-
Yeast	-	0. 5	-

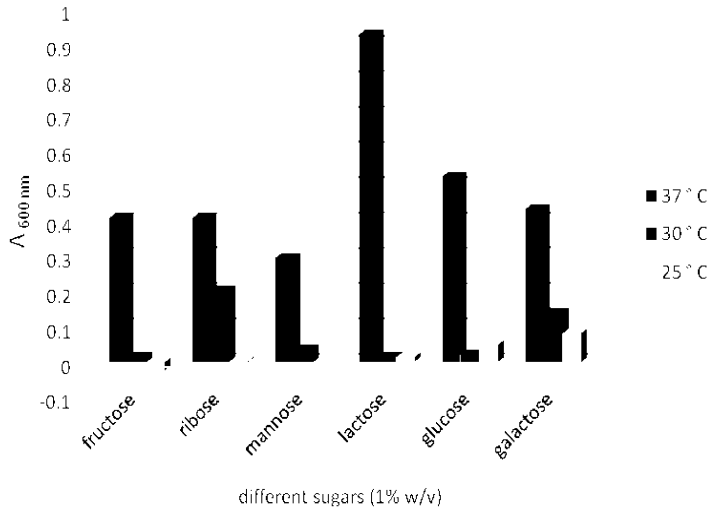


Fig. 1: Effect of temprature on EPS production in different carbohydrates (1% w/v) by *lactobacillus delbrueckii* spp *bulgaricus* incubated for 24 hr

production is affected by different species. Since EPS produced by microorganism are defined as biofilm formation by many authors so microtiter plate is used for EPS assay in this work. Compatibility of results obtained from this method with those of other conventional methods shows reliability of biofilm assay method in measuring EPS. Moreover due to presence of 96 wells in a microtiter plate allows conducting several experiments in the same time and under the same conditions. Similarity of the obtained results in these experiments shows repeatability of this method.

The Effect of Incubation Temperature on Biofilm Production: The effect of temperature (25°C, 30°C and 37°C) on biofilm production in *Lactobacillus delbrueckii* spp *bulgaricus* is shown in figure 1. The results showed that there was not any significant difference on biofilm production at 25 °C and 30 °C, however the significant effect at $p<5\%$ was observed at 37°C for *Lactobacillus delbrueckii* spp *bulgaricus*. This was observed in all isolated strains (data not shown). Optimum temperatures are reported to be 37, 42 and 45°C for production of EPS by *Lactobacillus delbrueckii* spp *bulgaricus*. There are different reports about the effect of temperature on EPS production in *Lactobacillus delbrueckii* spp *bulgaricus*. The difference in these reports is due to the use of different carbon sources and conditions however most authors obtained a range of 37-45° C as the optimum temperature for EPS production in *Lactobacilus delbrueckii* spp *bulgaricus* which is in accordance with this work (37°C). The range of (37-45°C) is also the optimum temperature for the growth of this microorganism. In some strains such as *Lactobacillus* strains the optimum temperature for growth leads to have optimum EPS production however in some strains this is not the case. The maximum temperature for EPS production in all isolated strains in this report was (37°C) (data are not shown). Higher temperature (45°C) significantly reduced biofilm formation in *Lactobacilus delbrueckii* spp *bulgaricus* figure 2A.

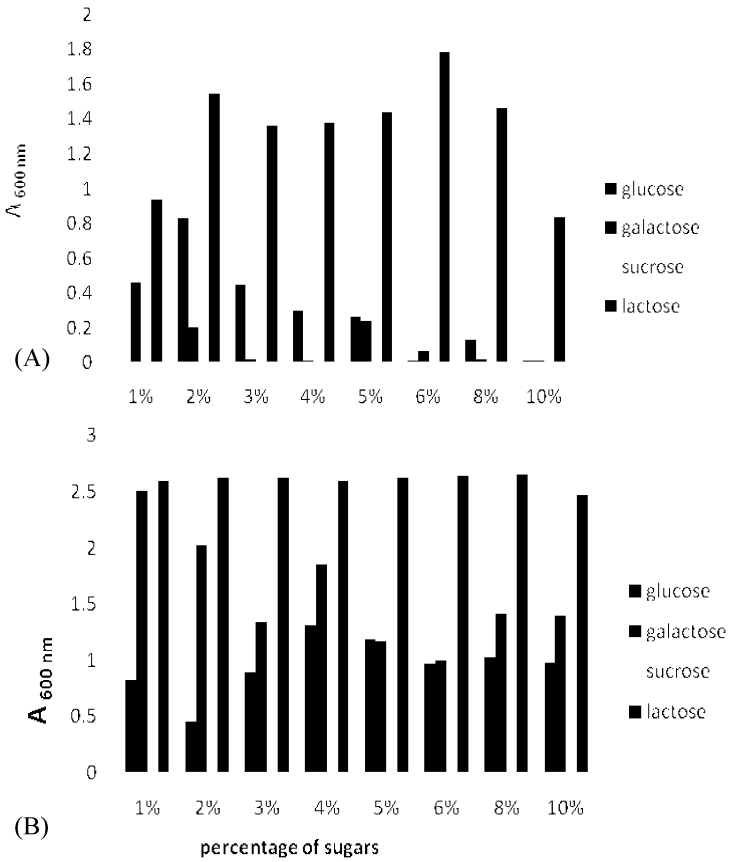


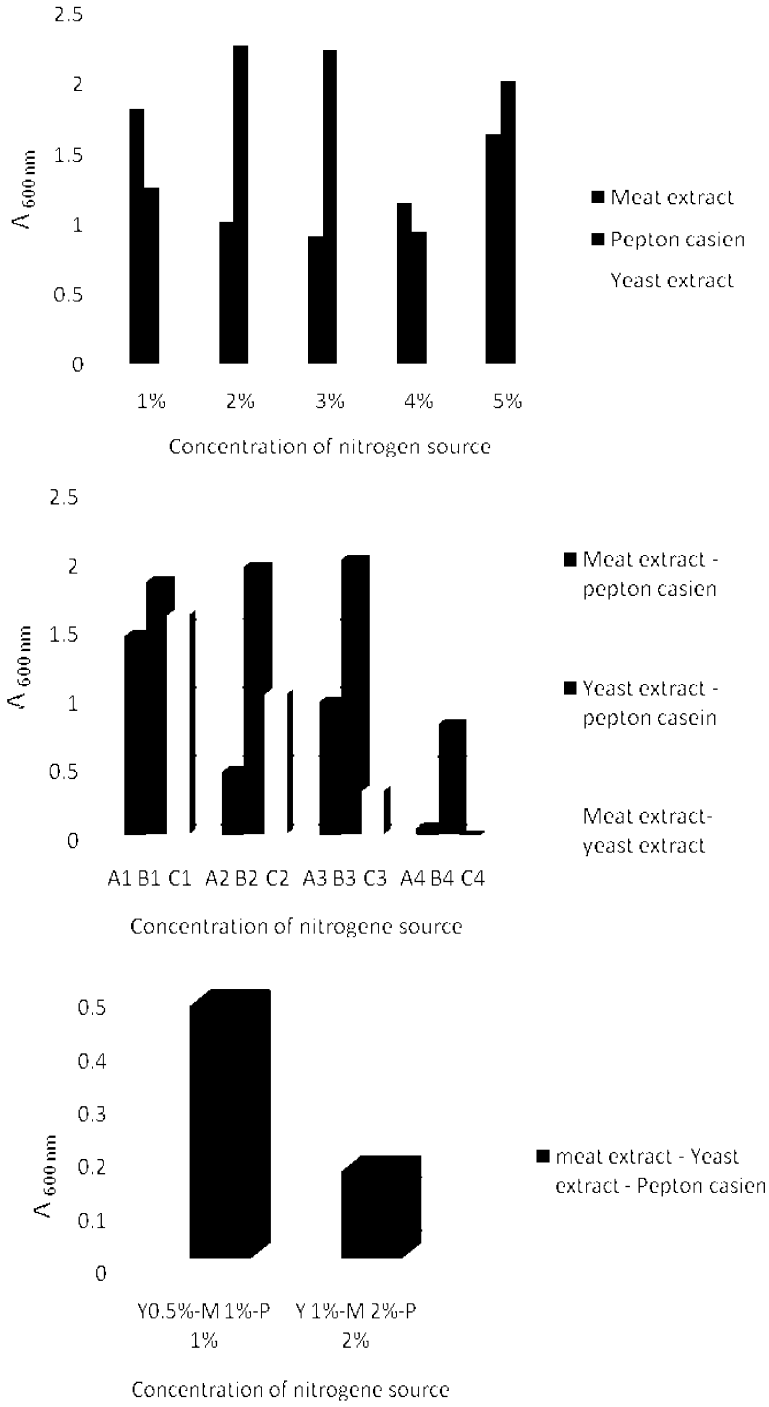
Fig. 2: Production of EPS by *lactobacillus delbrueckii* spp *bulgaricus* in different media with different carbohydrate concentration as it is shown lactose and 1% galactose was the best concentration for maximum EPS production
A= incubated at 45°C
B= incubated at 37°C

Table 5: The effect of lactose concentration on biofilm formation by *Lactobacillus delbrueckii* spp *bulgaricus* during 16 hr incubation at 37°C. (The Data are meaning of three replicate).

Percentage of lactose	1%	2%	3%	4%	5%	6%	8%	10%
Absorbance at 600 $_{nm}$	0.90 ^{a}	1.4 ^{b}	1.32 ^{c}	1.35 ^{bc}	1.36 ^{bc}	1.72 ^{d}	1.20 ^{ac}	0.80 ^{a}

The Effect of Carbohydrate and Concentration of Carbohydrate on Biofilm Production: The effect of carbohydrate and concentration of carbohydrate on biofilm production are shown in figure 2. As it is shown the most effective carbohydrate for EPS production by *Lactobacilus delbrueckii* spp *bulgaricus* is lactose (These data are statistically significant at p<5%). However for *Entrococcus facium* and *Lactococcus lactic,* glucose and galactose are suitable carbohydrate sources in EPS production, respectively. These data suggested that the biofilm formed as EPS might be Kefiran (EPS produced in Kefir which is a polymer of galactose and glucose). Although sucrose is a cheap source of carbon but cannot be considered as a good carbohydrate source for kefiran production since low amount of EPS is produced on this carbohydrate. The effect of lactose concentration on biofilm formation by *Lactobacillus delbrueckii* spp *bulgaricus* at 37°C are shown in Table 5. As it is shown at 6% (W/V) lactose, after 16 hours incubation, the maximum biofilm is made.

Most literature reviewers have reported that lactose is the best carbohydrate for EPS production in *Latobacillus delbrueckii spp bulgaricus* [1, 7, 18]. The obtained data in this work by measuring the biofilm formation also lead to have the same results. Furthermore Ferengova *et al*. [1] and Yokoi *et al*. [7] have shown that substitution of lactose with sucrose, mannose or fructose reduces Kefiran production which is compatible with



P= Pepton casein, M= Meat extract, Y=Yeast extract
A1:P 1%- M1 %; A2: P 2%-M 2%; A3: P 2%- M 1%; A4: P1%-M1%; B1: P1% - Y 0.5%; B2:P2%- Y1%; B3: P3%-Y2%; B4: P4%-Y1%; C1: Y 0.5%- M1%; C2: Y1%- M2%; C3:Y2%-M3%; C4:Y 1%- M4%;

Fig. 3: The effect of nitrogen source on EPS production by *lactobacillus debrueckii* spp *bulgaricus* as it is shown 2% pepton produced maximum EPS. the mixture of two nitrogen source did not have significant effect .

current data. Although Yokoi found that 10% (W/V) lactose gives more EPS but in this work with formation of biofilm on microplate, 6% lactose was found to be the best condition for EPS production.

The Effect of Nitrogen Sources and Concentration on Eps Production: The results of EPS production in different nitrogen sources with different concentration are shown in figure 3, 4 and 5. The results showed that *Lactobacillus delbrueckii* produced maximum EPS at 2% and 3% casein peptone and addition of 2% yeast extract had positive effect on the EPS production. Combination of peptone and yeast extract as nitrogen sources did not increase the EPS production. This concentration of peptone for EPS production is twice times more than that of required for growth (1% nitrogen source used in MRS medium). The same results were obtained by Yokoi, however Kimmel *et al.* [7, 13] produced maximum EPS from *Lactobacillus delbrueckii* in pH =5 at 37°C with addition of 3% bactocaseine as the nitrogen sources. They have shown that addition of 4% bactocasein inhibited the EPS production.

In conclusion, the study of biofilm formation on microplates is fast, cheep, repeatable and accurate method for study on EPS production of microorganism in pure cultures and it gives the same results that have been obtained by other methods. In this work it was found that *Lactobacillus delbrueckii* spp *Bulgaricus* isolated from kefir was the best EPS producer strain and produced maximum polysaccharide at 37°C with addition of 6% lactose and 2% peptone casein. Moreover *Lactococcus lactis* and *Entrococcus facium* produced EPS in galactose and glucose respectively and there was no EPS production observed in yeast and *Acetobacter*.

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