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**EFFECT OF INCUBATION PERIOD AND GROWTH MEDIUM COMPOSITION ON
EXOPOLYSACCHARIDES YIELD WITHIN INDIGENOUS FERMENTED KENYAN
*MURSIK MILK***

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ABSTRACT

The effect of incubation period and growth medium on Exopolysaccharides (EPS) yield from a traditionally fermented Kenyan milk (*Mursik*) culture was studied to determine the optimum conditions for EPS production. EPS yield levels were monitored over a 5 day period in four different growth media as: Reconstituted skim milk (RSM), RSM with 5% sucrose (RSM-S), Whey (WH) and WH with 5% sucrose (WH-S) at $20 \pm 1^\circ\text{C}$. The study established that the optimum incubation period for EPS production in traditional *Mursik* occurred after 4 days with an average yield of 73 mg/L Gluc. Equiv. The mean EPS level in all the growth media differed significantly ($p < 0.05$), and they ranked from the highest to the lowest as; RSM-S > WH-S > WH > RSM. Also, except for 18 and 24 hrs, the mean EPS levels for all the incubation periods differed significantly ($p < 0.05$), and ranked from the highest to the lowest as; 24hrs > 18hrs > 30hrs > 12hrs > 6hrs. The growth medium/incubation period interaction which yielded maximum EPS level (141.5 mg/L Glu. equiv) from *Mursik* culture was RSM-S/ 24 hrs. It was concluded that a four day incubation period was optimal for EPS levels in traditional *Mursik*.

EPS production within the *Mursik* culture is enhanced by inoculating the culture in milk that is supplemented with 5% sucrose.

Keywords: Exopolysaccharides, Optimization, Sucrose, *Mursik*

INTRODUCTION

Mursik is a Kenyan traditional fermented milk product that is produced by the Kalenjin community by spontaneous fermentation of cow milk [1]. It is thought to impart health beneficial effects to the consumers [2]. Exopolysaccharides (EPS) and EPS producing LAB which are mainly predominated by the *Lactococcus* species are thought to be the components which contribute to the beneficial health effects.

Exopolysaccharides (EPS) are polysaccharides that are excreted by microorganisms onto the surface of their cell walls as cohesive layers (capsular EPS) or into the growth medium as slime (ropy) EPS [3, 4]. A wide variety of microorganisms including moulds, yeasts, bacteria and algae have been reported to produce these EPS [3, 4]. They greatly vary based on monomer composition, molecular mass and structure [5, 6]. Production of EPS by Lactic acid bacteria species (LAB) has attracted a lot of interest in the food industry since they have unique properties and the LAB have 'generally regarded as safe' (GRAS) status [5]. They play an important role in improving rheological properties of fermented milk

products such as yoghurt and cheese as biothickeners, texturizers, stabilizer, gelling agents and water holding agents [7, 8]. They are preferred over the texturizers from the plant and algae sources, especially those that are chemically modified and those from non-food grade microorganisms such as xanthan and gellan [11]. The major limitation for use of LAB EPS as a bioingredient has been their low production capacities [12]. Most LAB produce below 1g/Liter EPS, whereas the non-food grade microorganisms such as *Xanthomonas campestris* produce as high as 30 to 50 g/Liter [13].

Several LAB EPS have also been established to have health beneficial effects to the consumer which includes the EPS with prebiotic or bifidogenic properties such as oligofructans [14], glucooligosaccharides and β -glucan [15]. Other health beneficial effects reported for EPS include the ability to reduce blood cholesterol levels, anti-carcinogenic [3] anti-temporal and immunomodulation activity [18, 19]. LAB EPS enhance the attachment and colonization of the gut by probiotics and this antagonizes the effects of pathogenic microorganisms. *Lactobacillus rhamnosus*

RW-9595M produces EPS which increase production of the cytokine IL-10 by the macrophages, and these prevent development of inflammatory conditions (immune-suppression) in the gut [18, 19].

Optimum EPS yields by most LAB are obtained at close to pH 6. In addition, it has been reported that the EPS yields are generally higher in pH-controlled systems than in un-controlled systems [20, 21]. Varying conditions in the culture during fermentation results in variation in EPS yields and composition, this is mainly because the changes result in different sugar activation and inter-conversion enzymes, which results in switching on or off certain enzymatic activities [22-25]. This study thus aimed to establish the effect of growth medium composition and incubation period on EPS production in *Mursik*. This information will be important in determining optimum conditions for EPS production in *Mursik*.

MATERIALS AND METHODS

The study was carried out at Egerton University (Kenya) at the Department of Dairy and Food science and Technology, which is located, between Longitudes 35° 45' and 35° 46' (East) and Latitudes 0° 16' and 1° 10' (South). Gourds were obtained from households in the study area. The gourds selected were those that had been ripened for

Mursik preparation in the homes. Raw milk was obtained from Guildford Dairy institute (GDI) dairy plant, at the Department of Dairy and Food science and Technology, Egerton University. '*Mursik culture*' (three days old *Mursik*) was obtained from a house hold in Belbur Village (Njoro District, Nakuru county) and kept in the fridge at 4°C before being used for the analysis. Dried skim milk powder was purchased from a local supermarket in Nakuru Town. Mesophilic milk culture (CHN-22, DVS) was a product of Chr Hansen (Denmark) and was Purchased from Promaco limited (Kenya). The experiment was carried out in a Completely Randomized Design. EPS yield in traditional *Mursik* was determined at 5 incubation periods (1, 2, 3, 4, 5 days) during traditional fermentation process. Also, EPS yield in four different growth media; Reconstituted Skim milk (RSM), RSM with 5% sucrose, Whey (WH), Whey with 5% sucrose (WH-S) and at 5 incubation periods (6, 12, 18, 24 and 30 hours) during incubation at 20± 1°C for 30 hours. Mesophilic milk culture was used as a control. The data obtained was used to compute for Analysis of Variance (ANOVA) using SAS statistical package (SAS system for windows, v.6.12, USA).

The fermentation gourds were cleaned and dried one day before *Mursik* preparation.

They were then applied charcoal fines from burned dry stems of *Senna didymobotrya* species ('Senetwet' in Kalenjin) using a bow shaped palm branch and left to cool for about 4 hours before filling the milk. Good quality raw milk, which met minimum acceptable quality specifications as (free from objectionable odour based on Organoleptic test, alcohol negative (using 72% ethanol) and with specific density of 1.028 to 1.032 (based on lactometer test) was used. The milk was tested for presence of inhibitory substances by starter culture activity test.

Mursik Fermentation

Mursik fermentation was carried by following the traditional procedure that is used by the Kalenjin community in Njoro District. Raw milk was heated to the boiling point, cooled to 20 ± 1 °C and then filled into the smoked gourds. The gourds were properly corked, labeled and incubated for 5 days at room temperature (20 ± 1 °C) in a box. During incubation, the milk was sampled daily (every 24 hours) for 5 days for analysis of EPS yield, Titratable acidity, pH, and Lactic acid bacteria (*Lactobacillus* and *Lactococcus*) counts.

Exopolysaccharide yields in the *Mursik* samples were determined by isolating and quantifying the EPS using alcohol precipitation and Phenol-sulphuric acid

methods respectively as per the method described by Cerning *et al.*, [30]. Titratable acidity was determined as per AAOAC, 1990 and the pH and Temperature examined using a milk analyzer (Lactoscan MCC30, Bulgaria) as per the procedure described by the manufacture. Lactic acid bacteria (LAB) in each *Mursik* were enumerated using M17 agar (Himedia) for *Lactococcus* species and MRS agar (Himedia) for *Lactobacillus* and *Leuconostoc* species.

Sweet cheese whey was prepared at Guildford Dairy Institute plant, following the plant procedures for Gouda cheese manufacture. Good quality raw milk, meeting GDI minimum acceptable quality specifications and free from inhibitory substances (selected as earlier described in 2.3.1.2) was used. The milk was then pasteurized at 63 °C /30 minutes and cooled to 30 – 32 °C. Active Mesophilic Lactic acid bacteria starter culture (CH-22, Chr Hansen) was added at the rate of 2% and ripened for 30 minutes. Rennet (CHY-MAX[®] Powder Extra NB, Chr Hansen) was added as per the manufactures instructions (0.03 g/ Liters milk) and allowed to set for 45 minutes. The curd was cut into 5 mm cubes, allowed to heal for 10 minutes and then the whey/curd mixture was gently stirred while raising the temperature gradually to 35 - 38 °C in within 30 – 60 minutes. The curds

were separated from whey and filtered using a muslin filter cloth followed by centrifugation at 4930 x g for 15 minutes to obtain clear whey (Figure 2). The whey was divided into two portions, and then the first portion was added 5% sucrose (50g / Liter) to make Whey-sucrose (WH-S) medium and the second portion remained as plain whey (WH) medium. Each medium was distributed into 500 ml screw capped bottles, each 200 ml and sterilized at 121 °C for 15 minutes. They were allowed to cool to room temperature after which they were ready for inoculation.

Reconstituted skim milk (RSM) was prepared by dissolving 12.5% skim milk powder in warm distilled water at 45 °C. It was divided into two portions, and then one portion was added 5 % Sucrose (50g/Liter) to make Reconstituted skim milk with sucrose (RSM-S) and the second portion remained as plain RSM. Each medium was distributed into 500 ml screw capped bottles, each 200 ml, sterilized at 121 °C for 15 minutes and cooled to room temperature, after which they were ready for inoculation.

Mursik culture (three days old *Mursik* product) was used as *Mursik* culture. The LAB counts in the culture was 8.72 Log cfu/ml *Lactococcus* and 8.39 Log cfu/ml *Lactobacillus* counts when determined using M17 and MRS agar respectively. Mesophillic

fermented milk culture was used as a control medium and was composed of the freeze dried Mesophillic milk culture (CHN-22, DVS). It was first propagated by transferring into sterile RSM (consisting of 12.5% m/v skim milk powder (New K.C.C) in distilled water), incubating at room temperature (20 ± 1°C) for 16 hours, and then kept in the fridge at 4 °C until the next day when it was used. The *Lactococcus* count in the Mesophillic milk culture was 8.43 Log cfu/ml enumerated using M17 agar. Each growth medium (WH, WH-S, RSM, RSM-S) was inoculated with 5 % *Mursik* culture and incubated at 20 ±1 °C in the water bath for 30 hours. Also, 5% Mesophillic milk culture was inoculated into each growth medium (control samples) and incubated at similar conditions as the *Mursik* samples. All the samples were monitored after every 6 hours during incubation for LAB growth, EPS production and change pH.

The *Mursik* and control samples were each sampled and analyzed after every 6 hours for 30 hours and analyzed. The EPS yield, pH and Lactic acid bacteria counts in each sample were determined as earlier described.

Data Analysis

The data obtained was subjected to Analysis of Variance (ANOVA) test using SAS statistical package (SAS system for window v.6.12, USA) to establish whether there were

significant differences between the four growth media (RSM-S, WH, WH-S and RSM), the five incubation periods (6, 12, 18, 24 and 30 hours).

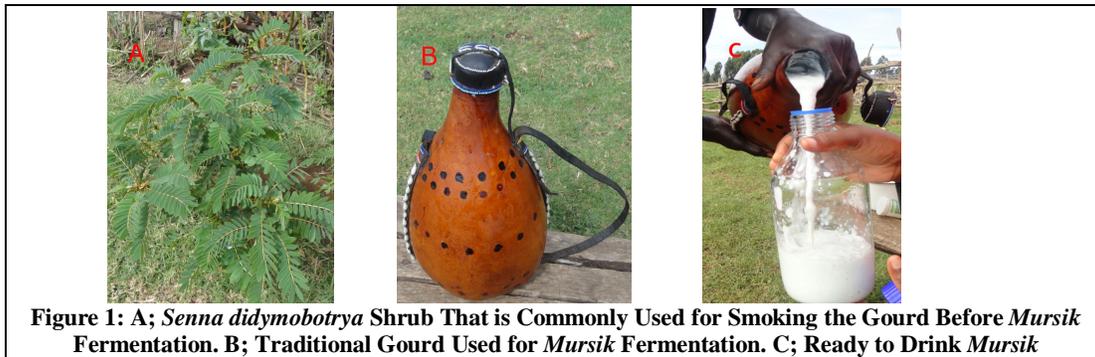


Figure 1: A; *Senna didymobotrya* Shrub That is Commonly Used for Smoking the Gourd Before *Mursik* Fermentation. B; Traditional Gourd Used for *Mursik* Fermentation. C; Ready to Drink *Mursik*



Figure 2: Whey (Cream-Yellow Coloured Liquid) in the Beaker Used for Preparation of the Whey and Whey Sucrose Media

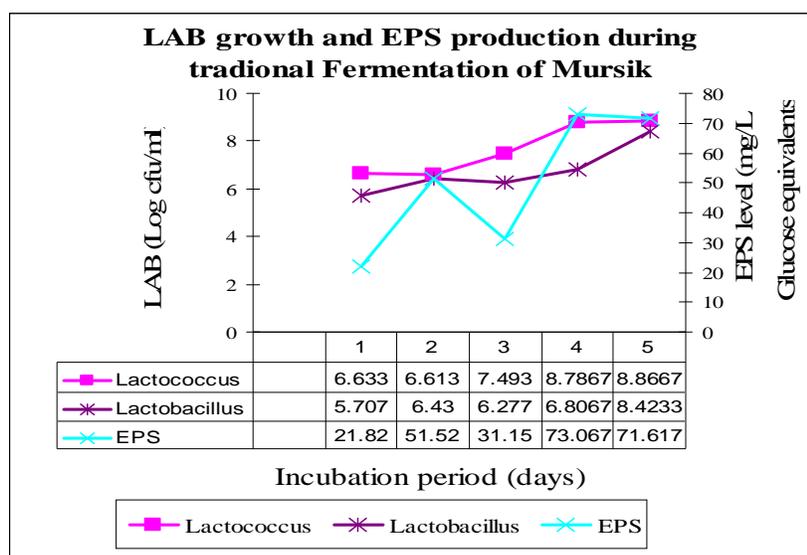


Figure 3: LAB Growth and EPS Production in *Mursik* During Traditional Fermentation Process

RESULTS AND DISCUSSION

Growth of both *Lactococcus* and *Lactobacillus* species during traditional fermentation of *Mursik* is shown in figure 3. Initially the LAB growth rate was generally low, between day one and two. However, the growth rate for *Lactococcus* increased between day two and four and reached stationary phase between day four and five. On the other hand the *Lactobacillus* growth rate remained slow up to day four when it started increasing at a faster rate up to day five. The *Lactococcus* population attained its maximum level of 8.8 log cfu/ml on day four whereas the *Lactobacillus* increased to 8.4 log cfu/ml by day five. The *Mursik* culture produced between 21.82 and 73.07 mg/L Gluc Equiv EPS during the 5 days incubation period. The EPS production was not growth associated. The levels were higher when the rate of LAB growth was low and vice versa. Its rate of production increased between day 1 and 2 after which it decreased on day 3 and increased again between day 3 and 4 to the maximum level of 73.1 mg/L Gluc.Equiv. The level went down slightly again on day 5 when the *Lactococcus* growth was at the stationary phase.

Change in Titratable acidity (T.A) and pH during traditional fermentation of *Mursik* is shown in **Figure 4**. The T.A increased

steadily from 0.21 % to 1.02% Lactic acid (L.A) during the 5 days incubation period, whereas the pH steadily decreased from pH 6.73 to pH 4.78. These changes were attributed to the breakdown of lactose (milk sugar) by the LAB resulting in production of lactic acid and other by-product such as carbon-dioxide and, acetic acid. These products increase in T.A/lowering of pH of the *Mursik* and this indicates active growth of the LAB.

The *Mursik* LAB (*Lactococcus* and *Lactobacillus*) species grew on all the four growth media, RSM-S, WH, WH-S and RSM. Their growth at 20 ± 1 °C for 30 hours followed the normal sigmoid growth curves as shown in **Figure 5**. In all the media, LAB growth was initially slow at between 6 and 12 hours, but after 12 hours, Logarithmic growth occurred and the *Lactococcus* species increased faster to a level slightly above that of the *Lactobacillus* species by the 18th hour (the end of logarithmic growth phase). After the 18th hour, the LAB entered the stationary phase (between 18 and 30 hours). The *Lactobacillus* species dominated the culture at between 6 and 12 hours of incubation, after which a shift of the dominant species occurred, and the *Lactococcus* species dominated the culture at between 18 and 30 hours (**Figure 5**). The *Mursik* culture

(inoculums) had 8.72 Log cfu/ml *Lactococcus* and 8.39 Log cfu/ml *Lactobacillus* counts at the time of inoculation into the growth media. This implies that although *Lactococcus species* were slightly more than the *Lactobacillus species* in the culture at inoculation time, their growth rate was initially lower compared to that for *Lactobacillus species*.

Mursik culture produced different EPS yields on the different growth media during the 30 hours incubation period and were as follows; in RSM-S, it ranged between 35.95 and 141.5 mg/L Gluc Equiv, in WH-S was between 30.38 and 55.75 mg/L Gluc Equiv, in RSM was between 22.64 and 37 mg/L Gluc Equiv and in WH was between 14.2 and 78.13 mg/L Gluc Equiv. RSM-S was the most superior medium for EPS production (**Figure 5**) by the *Mursik* culture, the optimum yield being 141.5 mg/L Gluc. Equiv. As earlier observed, the EPS yield in this medium increased at a higher when the rate of LAB growth was low and vice versa. The EPS levels increased steadily from 35.95 to 126.6 mg/L Gluc. Equiv at between 6 and 12 hours, when the LAB growth rate was low, and slightly decreased to 107 mg/L Gluc. Equiv at 12 and 18 hours when LAB growth were at the logarithmic phase. The levels increased again to 141.5 mg/L Gluc. Equiv (Maximum level)

at between 18 and 24 hours when LAB growth was at the stationary phase. The EPS levels again slightly decreased to 138.5 mg/L Gluc. Equiv at between 24 to 30 hours, when LAB growth was towards the end of stationary phase. WH, WH-S and RSM did not favour much EPS production but favoured normal LAB growth.

The growth of the LAB in Mesophilic milk culture (control) during the 30 hours incubation period followed a normal sigmoid curve pattern (**Figure 6**) as that for the *Mursik* LAB culture. Also, EPS yields differed in the four growth media as it occurred with *Mursik* culture, but the levels were generally low compared to that for *Mursik* culture. The EPS yield (in mg/L Gluc. Equiv) ranged between 7.2 and 47.3 mg/L in RSM-S, 16.2 and 65 mg/L in WH-S, 5.9 and 60.1 mg/L in RSM, and 10.2 and 47.5 mg/L in WH. The most superior medium for the EPS production by Mesophilic milk culture was WH-S, with a maximum yield of 65 mg/L Gluc.Equiv. Unlike the *Mursik* culture, EPS production in WH-S by Mesophilic milk culture occurred steadily regardless of the LAB growth rate, and the maximum level was attained at 24 hours. A similar trend was also observed in RSM-S, WH and RSM growth media as shown in **Figure 6**. The EPS yields by both *Mursik* and Mesophilic milk culture

decreased in all the four media when the LAB growth was towards the end of the stationary phase (24 and 30 hours). Generally, *Mursik* culture produced higher EPS yields in all the four growth media compared to Mesophillic milk culture.

Change of pH by *Mursik* and Mesophillic Milk Culture on Different Growth Media

The change in pH by *Mursik* and Mesophillic milk cultures during incubation (0 -30 hours) in all the four media (**Figure 7**) was uniform. The pH steadily dropped from pH 6.10 to pH 4.20 by *Mursik* culture and from pH 5.95 to pH 4.15 by Mesophillic milk culture. The EPS production experiments were carried out in uncontrolled pH conditions, which is a common condition for many traditional food fermentation processes. The pH drop indicates that the LAB cultures grew in the four different growth media.

Effect of Growth Media and Incubation Period on the Exopolysaccharide Yield by *Mursik* Culture

Results from analysis of variance (ANOVA) indicated that the four growth media (RSM-S, WH, WH-S and RSM), the five incubation periods (6, 12, 18, 24 and 30 hours) and the two cultures (*Mursik* and Mesophillic milk) were significantly different in EPS production at 5 % level of significance ($P < 0.05$). The

specific variations based on LSD ($P < 0.05$) are shown in the following results.

Effect of the Culture

The mean EPS yields by *Mursik* and Mesophillic milk (control) culture are shown in **Table 1**. There was a significant difference between the average EPS yield from *Mursik* culture and that from Mesophillic milk culture ($P < 0.05$). The EPS yield from *Mursik* culture was higher than that from Mesophillic milk culture, the average levels being 53.98 mg/L and 30.73 mg/L Gluc. Equiv. respectively.

Effect of Growth Media

The average EPS yields from the four growth media were all significantly different ($P < 0.05$). Based on LSD test, all the growth media significantly differed from each other as shown in Table 2. RSM-S and WH-S, both of which were supplemented with 5 % sucrose produced higher EPS yields, the average being 68.7 and 44.6 mg/L Gluc.Equiv respectively, whereas RSM and WH which were not supplemented with sucrose produced low EPS yields (average of 30.5 and 25.7 mg/L Gluc.Equiv respectively). This clearly shows that sucrose stimulated EPS production by both the *Mursik* and Mesophillic fermented milk cultures. The average EPS yield for the four growth media when ranked from the highest to the lowest was as follows; RSM-S > WH-S > WH > RSM.

Effect of the Incubation Period

The EPS yields from the five incubation periods were significantly ($P < 0.05$). Based on LSD test all incubation periods except 18 and 24 hours were significantly different as shown in **Table 3**. Generally, the EPS levels increased with increase in incubation period from 6 hours to the optimum period at 24 hours, after which they started decreased as observed after 30 hours. It was clear that shorter incubation periods (6 and 12 hours) produced lower EPS levels than longer incubation periods (18 – 30 hours). However, the EPS yields produced at 18 and 24 hours were higher than the yields at 30 hours. This implies that prolonged incubation period resulted in decrease in the EPS yield. The incubation periods which produced optimum EPS yields were 18 and 24 hours, average yields being 52.8 and 56.7 mg/L Gluc.Equiv respectively, and both were not significantly different. Based on average EPS yield, the incubation periods can be ranked from the highest to the lowest level as follows; 24 hours > 18 hours > 30 hours > 12 hours > 6 hours.

Interactions Between Culture and Growth Medium

There was a significant difference between the different interactions between culture and growth media ($P < 0.05$). From the results, it

was evident that the interaction between the growth medium and culture influenced EPS production by both *Mursik* and Mesophillic milk cultures as shown in **Table 4**.

Both cultures (*Mursik* and Mesophillic milk cultures) produced higher EPS yields in the media supplemented with sucrose (RSM-S and WH-S). However, Culture/Growth medium (interaction) which produced the best EPS yield was *Mursik*/RSM-S with an average yield of 109.9 mg/ L Gluc.Equiv. this was followed by Mesophillic milk culture/WH-S with average yield of 45.3 mg/L Gluc.Equiv. While *Mursik* culture produced its optimum yield in RSM-S Mesophillic milk culture (Control) produced its optimum yield in WH-S. This implies that, both cultures varied in their growth media requirements for EPS production. The EPS yield by *Mursik* culture in the four growth media can be ranked from the highest to the lowest as follows; RSM-S > WH-S > WH > RSM, whereas for Mesophillic milk culture, the highest yield was from WH-S, and RSM-S, WH and RSM produced similar EPS yields (were not significantly different at $P < 0.05$). It was also observed that the interaction between *Mursik* culture and the other growth media produced higher EPS yields compared to the interaction with Mesophillic milk culture (**Table 4**). The best culture/growth

interaction was *Mursik*/RSM-S, with an optimum yield of 109.9 mg/L Gluc.Equiv.

Interaction Between Culture and Incubation Period

Average EPS yield for the interaction between incubation period and culture is shown in Table 5. There was a significant difference between the interaction between incubation periods and cultures ($P < 0.05$). For *Mursik* culture, the average EPS yield at all incubation periods except 18 and 24 hours significantly differed. The optimum EPS yield for *Mursik* culture occurred at 18 -24 hours, average yields being 66.0 and 60.8 mg/L Gluc.Equiv respectively. For Mesophillic milk culture, the average EPS yields at all the incubation periods were significantly different, and the optimum yield (52.5 mg/L Gluc.Equiv) was at 24 hours. For *Mursik* culture, The incubation periods can be ranked from the highest to the lowest based on average EPS yields as follows; 18 hours > 24 hours >30 hours >12 hours > 6 hours, whereas for Mesophillic milk culture, they can be ranked as follows; 24 hours > 18 hours > 30 hours > 12 hours > 6 hours. In both cultures, 6 and 12 hours produced generally low yields and prolonged incubation (30 hours) resulted in decrease in the EPS yields. Generally, it was observed that the interaction between all incubation periods

with *Mursik* culture resulted in higher EPS yields than their interaction with Mesophillic milk culture. Optimum EPS yield occurred from the interaction between *Mursik* culture and 18 hour incubation period, the average yield being 66 mg/L Gluc.Equiv. This was not significantly different with the yield from the interaction between *Mursik* culture and 24 hour incubation period (60.8 mg/L Gluc Equiv).

Interaction between Incubation Period and Growth Media

Effect of the interaction between growth medium and incubation period on EPS yield is shown in **Table 6**. There was a significant difference in the average EPS yields due to the interactions between incubation periods and growth media ($P < 0.05$). The optimum EPS yield in the different growth media occurred at different incubation periods, mainly between 18 and 24 hour. The average EPS levels during the 30 hours incubation period for the different growth media ranged between 21.59 and 94.38 mg/L in RSM-S (optimum being at 24 hours), between 23.28 and 54.84 mg/L in WH-S (optimum being at 18 – 24 hours), between 12.21 and 62.81 mg/L in WH (Optimum being at 18 hours), and between 14.25 and 48.54 mg/L in RSM (optimum being at 24 hours).

RSM-S produced higher EPS levels compared to WH-S, WH and RSM at 12 to 30 hour incubation periods and was followed by WH-S. This indicates that, the sucrose supplementation improved the EPS yields. Based on EPS yields, the incubation periods for each growth medium can be ranked from the highest to the lowest as follows; RSM-S, 24 hours > 30 hours > 12 hours > 18 > 6 hours, where 24 and 30 hours, and 12 and 18 hours were not significantly different; WH, 18 hours > 24 hours > 30 hours > 12 hours > 6 hours, where 24 and 30 hours, and 6 and 12 hours were not significantly different; WH-S, 24 hours > 30 hours > 18 hours > 12 hours > 6 hour, where 18, 24 and 30 hours were not significantly different; and RSM, 24 hours > 18 hours > 12 hours > 30 hours > 6 hours, where 6 and 30 hours were not significantly different. In all the growth media, 6 to 12 hours produced lower EPS levels whereas 30 hours resulted in reduction of the EPS yields. The growth medium/incubation period interaction which produced the highest EPS yield was RSM-S/24 hours, with the average EPS yield of 94.4 EPS mg/L Gluc.Equiv. Prolonged incubation period (30 hours) resulted in reduction of the EPS level to 85.1 mg/L Gluc.Equiv but the level was not significantly different from the optimum level.

Interaction Between Culture, Growth Medium and Incubation Period

The effect of the interaction of all the three factors, culture, growth medium and incubation period on EPS yields are shown in **Table 7**. There was a significant difference between the mean EPS levels for the three factor interactions (culture/Growth medium/Incubation period) at $P < 0.05$. The interaction between *Mursik* culture and RSM-S produced higher EPS yields at all incubation periods compared to its interaction with WH-S, WH and RSM. Also, both *Mursik* and Mesophilic milk culture produced higher EPS yields in WH-S compared to WH and RSM. This implies that sucrose supplementation stimulated EPS yields by both *Mursik* and Mesophilic milk culture. However, *Mursik* culture produced higher EPS levels in all the growth media compared to the Mesophilic milk culture. Optimum yields in both *Mursik* and Mesophilic milk culture were obtained at 24 and 18 hours incubation periods respectively, whereas at 30 hours, EPS reduction was observed. *Mursik* culture/ RSM-S/24 hours produced the highest EPS yield (average yield being 141.5 mg/L Gluc.Equiv). These observations are in agreement with the earlier observation, for the interaction between *Mursik* culture and incubation period (18 - 24 hours) in **Table 5**,

and the interaction between RSM-S and 24 hour incubation period in **Table 4** which produced the highest EPS yields.

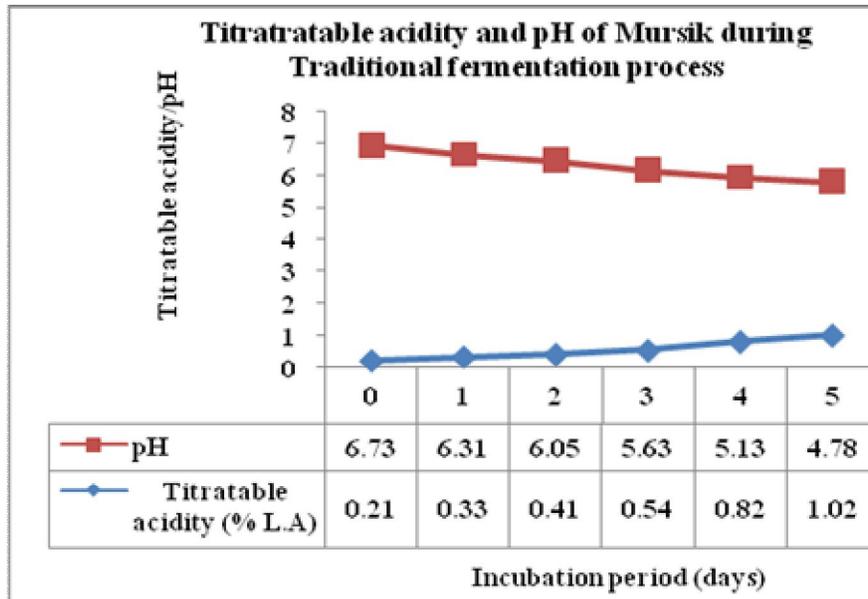


Figure 4: Titratable and pH of *Mursik* During Traditional Fermentation Process

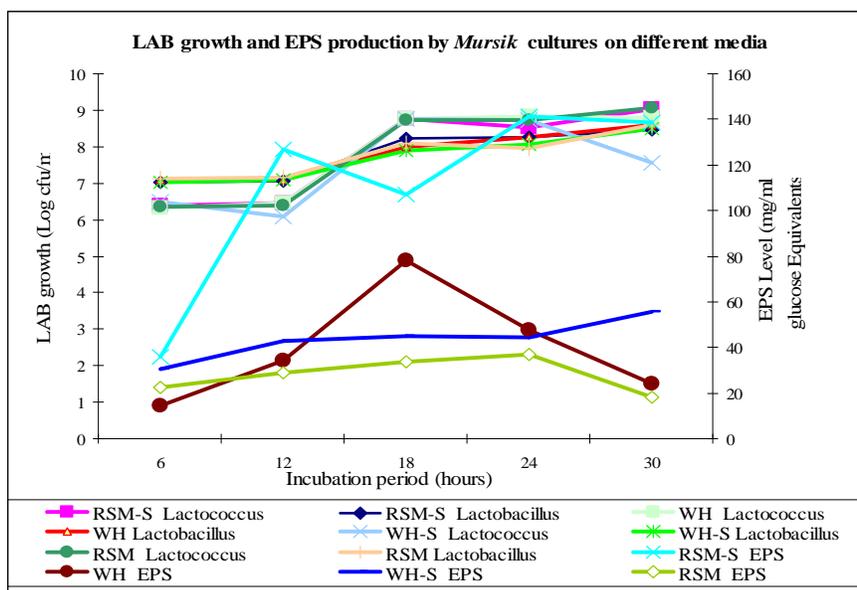


Figure 5: LAB growth and EPS Production by *Mursik* Culture in Different Growth Media

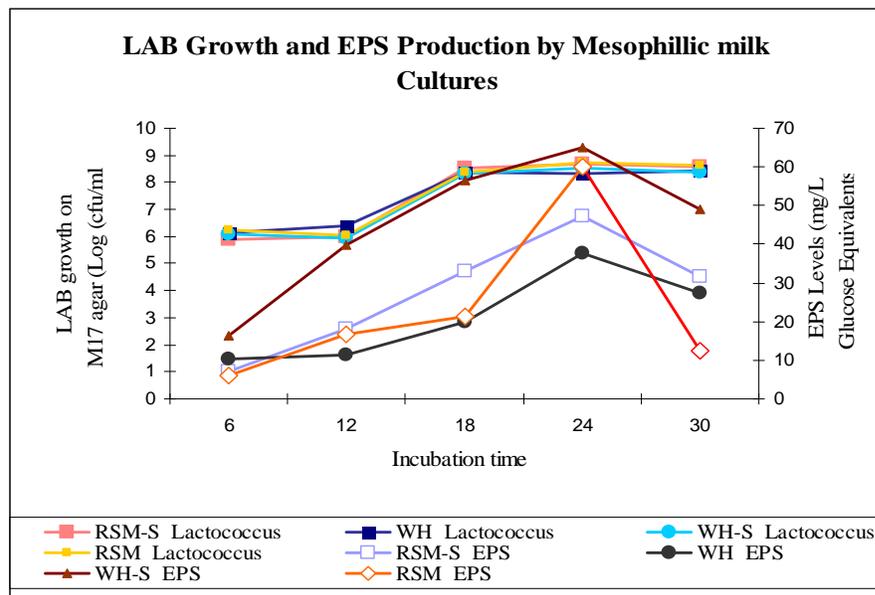


Figure 6: LAB Growth and EPS Production by Mesophilic Milk Cultures

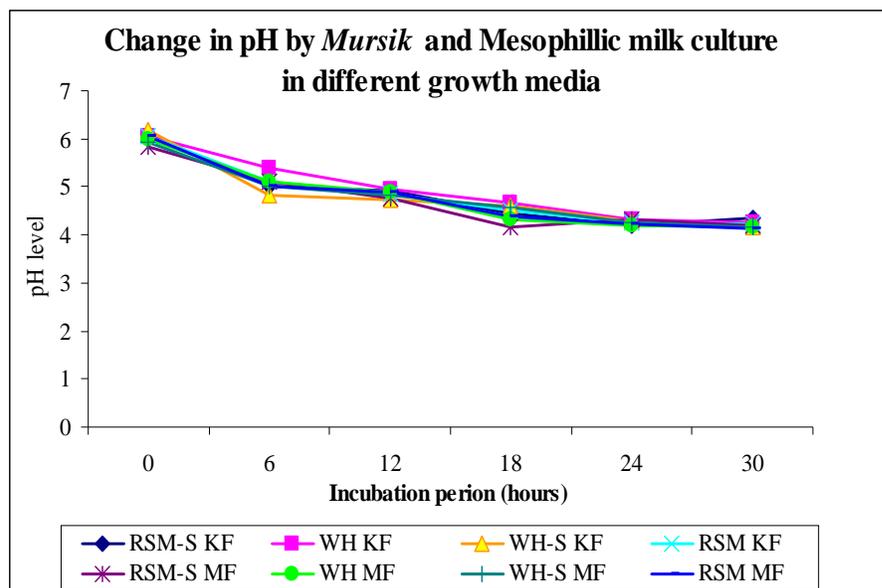


Figure 7: Change in pH in Different Growth Media During Growth of the Mursik Culture

Table 1: Average Levels of Exopolysaccharides From *Mursik* and Mesophilic Milk Culture

Culture	Mean EPS (mg/L Glucose Equivalents)	SD
<i>Mursik</i>	53.98 ^a	± 40.97
Mesophilic fermented milk	30.73 ^b	±18.87

Note: Means with the same letter are not significantly different at $P < 0.05$. (LSD = 2.6917, n = 40)

Table 2: Average Levels of Exopolysaccharides on Different Growth Media

Growth medium	Mean EPS (mg/L Glucose Equivalents.)	SD
Reconstituted skim milk with 5 % sucrose (RSM-S)	68.7 ^a	± 52.18
Whey with 5 % Sucrose (WH-S)	44.6 ^b	± 14.15
Whey (WH)	30.5 ^c	± 20.28
Reconstituted skim milk (RSM)	25.7 ^d	± 15.28

Note: Means with the same letter are not significantly different at $P < 0.05$. (LSD test = 3.8067, n = 20)

Table 3: Average Levels of Exopolysaccharides Produced by *Mursik* and Mesophilic Milk Cultures After Different Incubation Periods

Incubation Time (Hours)	Mean EPS (mg/L Glucose Equivalents)	SD
24	56.7 ^a	± 35.99
18	52.8 ^a	± 27.65
30	44.6 ^b	± 39.60
12	39.9 ^c	± 35.75
6	17.8 ^d	± 10.81

Note: Means with the same letter are not significantly different at $P < 0.05$. (LSD = 4.256) (n = 16)

Table 4: Average Exopolysaccharide Levels (mg/L Glucose Equivalents) for the Interaction Between Growth Medium and Culture

GROWTH MEDIUM	<i>Mursik</i> Culture		Mesophilic milk Culture	
	Mean EPS	SD	Mean EPS	SD
RSM-S	109.94 ^a	± 41.737	27.51 ^b	± 15.221
WH	34.13 ^c	± 24.439	26.83 ^b	± 15.546
WH-S	43.78 ^b	± 10.204	45.32 ^a	± 17.811
RSM	28.10 ^d	± 7.383	23.26 ^b	± 20.621

Note: Means in the same column with the same letter are not significantly different at $P < 0.05$. (LSD= 5. 383, n = 4)

Table 5: Average Exopolysaccharide Levels (mg/L glucose Equivalents) for the Interaction Between Culture and Incubation Period

INCUBATION TIME (HOURS)	CULTURE			
	<i>Mursik</i>		Mesophilic milk	
	Mean EPS	SD	Mean EPS	SD
6	25.79 ^c	± 9.027	9.88 ^c	± 4.922
12	58.22 ^b	± 42.793	21.55 ^d	± 11.813
18	66.00 ^a	± 31.977	39.67 ^b	± 14.808
24	60.80 ^{ab}	± 50.787	52.51 ^a	± 12.549
30	59.12 ^b	± 51.587	30.03 ^c	± 14.685

Note: Means in the same column with the same letter are not significantly different at $P < 0.05$. (LSD = 6.019, n = 10)

Table 6: Average Levels of Exopolysaccharides (mg/L glucose Equivalents) for the Interaction Between Growth Medium and Incubation Period

Incubation Time (hrs)	Growth Medium							
	RSM-S		WH		WH-S		RSM	
	Mean EPS	SD	Mean EPS	SD	Mean EPS	SD	Mean EPS	SD
6	21.59 ^c	± 16.734	12.21 ^c	± 3.239	23.28 ^c	± 8.982	14.25 ^c	± 9.773
12	72.21 ^b	± 62.579	22.86 ^c	± 14.232	41.41 ^b	± 5.773	22.85 ^c	± 7.184
18	70.14 ^b	± 44.547	62.81 ^a	± 17.932	50.91 ^{ab}	± 8.081	27.48 ^b	± 7.513
24	94.38 ^a	± 54.630	28.89 ^b	± 10.641	54.84 ^a	± 11.801	48.54 ^a	± 14.931
30	85.10 ^a	± 62.137	25.60 ^b	± 2.557	52.31 ^{ab}	± 7.599	15.28 ^c	± 4.676

Note: Means in the same column with the same letter are not significantly different at $P < 0.05$. (LSD = 11.6, n = 8)

Table 7: Average Levels of Exopolysaccharides (mg/L glucose equivalents) for the Interaction Between Growth Medium, Incubation Period and Culture

Incubation Time (hrs)	<i>Mursik Culture</i>				Mesophilic milk Culture			
	RSM-S	WH	WH-S	RSM	RSM-S	WH	WH-S	RSM
6	35.95 ^d (±2.333)	14.20 ^c (±2.404)	30.38 ^c (±4.632)	22.64 ^{bc} (±0.707)	7.23 ^c (±3.076)	10.23 ^c (±3.147)	16.18 ^d (±4.349)	5.88 ^c (±2.369)
12	126.60 ^b (±0.990)	34.30 ^b (±8.132)	42.95 ^b (±8.980)	29.02 ^{abc} (±1.591)	18.23 ^c (±1.502)	11.43 ^c (±4.278)	39.88 ^c (±3.147)	16.68 ^{bc} (±0.177)
18	107.08 ^c (±21.248)	78.13 ^a (±3.571)	45.15 ^{ab} (±7.920)	33.65 ^{ab} (±0.071)	33.20 ^b (±6.647)	47.50 ^a (±3.748)	56.68 ^{ab} (±0.601)	21.30 ^b (±4.101)
24	141.50 ^a (±5.374)	20.05 ^c (±3.677)	44.65 ^{ab} (±1.018)	37.0 ^a (±3.246)	47.25 ^a (±6.435)	37.73 ^{ab} (±3.712)	65.00 ^a (±1.626)	60.08 ^a (±11.028)
30	138.55 ^{ab} (±16.435)	23.95 ^b (±2.828)	55.75 ^a (±10.889)	18.22 ^c (±0.007)	31.65 ^b (±10.677)	27.25 ^b (±0.849)	48.88 ^{bc} (±2.722)	12.35 ^{bc} (±5.586)

Note: Means with the Same Letter in the Same Column are not Significantly Different at $P < 0.05$ (LSD= 12.04, n = 2)

DISCUSSION

Mursik is a product of spontaneous fermentation that is composed of mixed culture which naturally comes from the fermentation container, gourd, and milk although the main fermentation microorganisms in traditional *Mursik* are LAB [4]. In this study, production of EPS in *Mursik* during traditional fermentation process was observed to be non-growth associated. Higher EPS levels were produced when LAB growth rate was low and vice versa. The EPS level increased steadily between day one and day two (when LAB growth rate was low), after which they rapidly decreased between day two and three (when the LAB, (*Lactococcus species*) growth rate was logarithmic). Another rapid increase was observed between day three and four (when the LAB growth rate was in the stationary phase), after which they slightly decreased between days four and five. The rapid decrease of EPS levels observed between day two and three implies that other than retarded EPS biosynthesis, the initially formed EPS were also being broken down or lost in the *Mursik*. These observations were in agreement with those reported by [6] and [27] that EPS production in Mesophilic LAB occurs mainly when the rate of LAB growth is low. This is because in these LAB species,

biosynthesis of EPS and cell wall polymers (peptidoglycan, teichoic acid, lipoteichoic acid and others) occur in almost similar processes, hence they have similar requirements especially the sugar nucleotides, housekeeping enzymes and isoprenoid lipid carriers for assembly of the polymers [6, 25, 27]. Therefore, they produce higher EPS yields when the LAB growth rate is low since there is less demand for cell-wall polymers and so the sugar nucleotides, housekeeping enzymes and isoprenoid lipid carriers are diverted towards EPS biosynthesis [4, 25]. Based on this, propagation of Mesophilic LAB at sub-optimal growth conditions such as low temperatures and pH, favours production of higher EPS yields because at such conditions, bacterial cell growth is low [4, 25]. Decrease in EPS levels observed in *Mursik* during incubation (day 2 to 3 and day 4 to 5), was attributed to the breakdown of EPS polymers by EPS degrading enzymes (*glycohydrolases*) which may have been produced by some LAB. *Glycohydrolases* hydrolyze the EPS polymers in the culture resulting in decrease of the EPS yields [20] and this occurs mostly at the stationary growth phase or during prolonged incubation periods [20]. Production of EPS degrading enzymes vary with the LAB strain and is stimulated by certain growth conditions such

as temperature and pH [6, 20]. Therefore, these stimulatory conditions may have been created in the *Mursik* culture during fermentation since fermentation conditions such as pH were not controlled.

Exopolysaccharide (EPS) yields by the EPS producing LAB are influenced by several factors which include the LAB strain, composition of the growth media and growth conditions. Studies by [22] established that EPS producing LAB strains produce different types of EPS mainly based on monomer composition, yield and molecular mass. EPS production phenotype in the EPS producing LAB is controlled by the *eps* genes that they possess and they vary with the species and strains [22]. The *eps* genes determine the type of *glycosyltransferases* to be synthesized by the LAB strain, hence the type of EPS it produces [12]. In this study, it was established that both *Mursik* and Mesophilic milk culture (control) produced different EPS yields. This difference was attributed to variation in their LAB (culture) composition and their genetic potentials for EPS production. Basically, *Mursik* and Mesophilic milk cultures vary in their LAB composition, the *Mursik* being composed of a mixed LAB culture with *Lactococcus* and *Lactobacillus* species and the Mesophilic milk culture being composed

of only two strains of *Lactococcus lactis* strains [36].

Growth medium composition, mainly carbon and nitrogen sources, influence EPS production by LAB. Studies have shown that the nature and composition of the growth medium (carbon, nitrogen, mineral salts and vitamins) affect EPS production by a given LAB strain [23, 24]. Energy production is very essential during EPS biosynthesis since it is required for production of large numbers of sugar nucleotides, polymer formation, bacterial cell growth and other metabolic activities in the cell [14, 33]. Carbon (sugar) is required for synthesis of sugar nucleotides such as uridine diphosphate- glucose (UDP-glucose), UDP-galactose and deoxythymidine diphosphate-rhamnose (dTDP-rhamnose) which are the donors of the sugar monomers during biosynthesis of the bacterial polysaccharides, EPS and cell wall polymers [15, 27]. The nature and concentration of carbon source strongly influences EPS production by LAB [1, 26], but the most suitable sugar for EPS production mainly depends on the ability of the bacteria strain to utilize it [1, 25]. Some LAB produce high amount of EPS on one type of carbon source than on another. In this study, it was observed that *Mursik* culture produced higher EPS yields in the media that were supplemented

with sucrose (RSM-S and WH-S) than in the non-sucrose supplemented media (RSM and WH).

The main carbon source in the non-sucrose supplemented media was lactose whereas in the supplemented media it was mainly sucrose, also, in this study, EPS yields in the sucrose supplemented media varied between the two cultures (*Mursik* and mesophilic milk cultures). *Mursik* culture produced optimum EPS yields in RSM-S whereas Mesophilic milk culture produced its optimum EPS yield in WH-S. These clearly show that sucrose stimulated EPS production by *Mursik* culture much better than by the Mesophilic milk culture. Studies have shown that addition of sucrose into the growth media for EPS production increase EPS yields by certain EPS producing LAB species including *Lactobacillus sanfranciscensis* LTH2590 [13], *Weissella* species [35] and *Leuconostoc mesenteroides* [34]. Nitrogen sources (proteins) are also important nutrients for EPS production by LAB. Enough nitrogen is required for formation of the various cell components and enzymes required during EPS biosynthesis [20, 31]. Complex proteins are good nitrogen sources since they contain several peptides and amino acids that are important for both cell growth and EPS production [5, 6].

Dairy by-products such as whey and protein hydrolysates contain peptides and amino acids that are readily available for assimilation by the bacteria cells [5]. Whey concentrates, whey permeate and casein have been shown to improve EPS yields by LAB such as *Lactobacillus rhamnosus* [30], *Streptococcus thermophilus* [6] and *Lactobacillus delbrueckii bulgaricus* [23]. According to [1], LAB species have limited abilities to synthesize amino acids that are essential for their growth, but they can hydrolyze milk proteins to obtain them. Cheese whey is a by-product from cheese manufacture that consist of whey proteins, mainly lactalbumin and Lactoglobulin ($\approx 20\%$) [36]. It also contains the water soluble kappa-casein, glycomacropptide, (0.8 -1 % protein) that are formed after cleavage of kappa-casein by chymosin enzyme during cheese curd formation [36]. All this are proteins that the EPS producing LAB can derive nitrogen sources. However, the influence of the nitrogen source on EPS production depends on the LAB strain and its EPS production Kinetics. EPS production by most thermophilic LAB is growth associated hence their EPS products are primary metabolites whereas in Mesophilic LAB, EPS production is non-growth associated hence their EPS are secondary metabolites [25]. Since in growth

associated EPS production, the rate of EPS production is highest at the exponential growth phase, any approach that will increase LAB growth rate also increases the rate of EPS production [25]. However, in non-growth associated EPS production, where the rate of EPS production is high when the rate of LAB production is lowest, optimum EPS yield are obtained at sub-optimal growth conditions [25]. Therefore, in mesophilic LAB, any approach that will result in slow rate of bacterial growth will optimize EPS yield. Some LAB can produce different LAB at different stages of growth, one as a primary metabolite and another as a secondary metabolite [25]. In this case, nitrogen source will influence the composition of the primary metabolite EPS in the overall EPS yield [25]. Incubation period has been shown to affect EPS production by LAB. This was also observed in this study, both in *Mursik* and Mesophilic milk (control) cultures. It was established that shorter incubation periods (6 - 12 hours) were insufficient for EPS production whereas prolonged incubation periods (30 hours) resulted in decrease in EPS levels. Optimum incubation period for EPS production by *Mursik* culture was 18 - 24 hours, the best being 24 hours. Studies have shown that some EPS degradation occurs upon prolonged incubation of some LAB

cultures [20, 23] due to breakdown by *glycohydrolases* [20, 27]. According to [23] and [20] EPS degradation process is induced by certain environmental conditions around the cell, such as certain pH levels and temperature. However, some LAB species such as *Streptococcus thermophilus* ST 111 do not produce EPS degrading enzymes [5].

CONCLUSION

Supplementation of the milk with sucrose increases the EPS yields by *Mursik* culture and the optimum yield in the sucrose supplemented milk is obtained after incubation for 24 hours at $20 \pm 1^\circ\text{C}$. The EPS yields by *Mursik* culture can be improved by inoculating it in sucrose-supplemented (with 5 % sucrose) milk and incubating for 24 hours at $20 \pm 1^\circ\text{C}$. Prolonged incubation of the *Mursik*, over 4 days in traditional method and over 24 hours (culture method) results in reduction of the EPS yields.

The functional properties of *Mursik* should be improved by improving their EPS levels. This can be achieved by optimizing the EPS levels in the traditional *Mursik* by incubating them for 4 days during traditional fermentation process or by inoculating *Mursik* culture (backslipping) in milk that is supplemented with 5% sucrose and incubating for 24 hours. In the long term, it can be achieved by developing and preserving a defined *Mursik*

culture which can be used for production of *Mursik* using modern fermentation technologies which guarantee good quality and product safety, as well as enable application of technologies for improved EPS yield such as supplementation of *Mursik* milk with sucrose. However, more studies should be done in future to establish the effects of other sugars such as glucose, fructose, galactose, maltose among others, and other growth conditions such as other levels of temperature, pH and oxygen potential on the EPS yield by *Mursik* culture.

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