Research Article

# Identification and Characterization of Exopolysaccharides of *Staphylococcus epidermis* and *Staphylococcus capitis* Isolated from Goat Gut

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## ABSTRACT

The present work was delineated to isolate exopolysaccharide (EPS) producing lactic acid bacteria such as *Staphylococcus epidermis* and *Staphylococcus capitis.* The synthesis of exopolysaccharide was identified by growing the cultures in Congo red agar and visual identification of black colonies proved to have EPS production by both the isolates. The exopolysaccharides has been extracted and quantified. The characterisation of exopolysaccharide was done by SEM, EDAX and FTIR. The antioxidant activity of the exopolysaccharides has been performed and the results indicated that there was increasing inhibition with increase in concentration. The metal chelating assay resulted that ferrozine can quantitatively form complexes with $Fe^{2+}$. Thus EPS shows metal chelating activity. The emulsifying activity of the exopolysaccharides has been performed and the result proves that the EPS can be used as a potent bio emulsifier in food industries. Thus, the results suggested that the exopolysaccharides has potential application in pharmaceutical and food industries.

**Key words:** Exopolysaccharide, Antioxidant, Metal chelating activity, Emulsification, Lactic acid bacteria, Staphylococcus.

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## INTRODUCTION

Exopolysaccharides are high molecular weight, biodegradable polymers synthesized by various types of bacteria. Lactic acid bacteria (LAB) has an ability to produce exopolysaccharides.[1] Many researchers suggest that EPS produced by Latic acid bacteria are more beneficial to consumers. LAB is generally recognized as safe (GRAS) microorganisms and their ability to produce EPS have wide diversity of structures without health. EPS produced by LAB have recently got an increasing amount of attention because of their health benefits to the consumers.[2]

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Free radicals are unstable and they are deleterious to human. Elimination of these free radicals both synthesised and natural antioxidants are used however, synthetic antioxidants cause liver damage and carcinogenesis. Therefore, it is indispensable to develop a natural nontoxic antioxidant to protect humans from free radicals.[3] There are number of exopolysaccharides obtained from microorganisms which were found to possess potent antioxidant activities.[4] Exopolysaccharides have been considered to be propitious antioxidants that could be accepted as important candidates for the development of effective and non-toxic medicines with stronger antioxidant activities *in vitro* and *in vivo*[5]

The EPS synthesized from Lactic acid bacteria has various applications in various industries like food industry as stabilizing, viscousifying, jellying and emulsifying agent. The demand for bioemulsifiers has been increasing day by day, especially in food industries and personal care product manufacturing sectors.[6] Biosurfactants are surface active biomolecules produced

by microorganisms. These molecules are capable of reducing surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures. Biosurfactants with high molecular weight can produce stable emulsions without lowering surface or interfacial tension and they are called bioemulsifiers. Bioemulsifiers have higher biodegradability over chemical emulsifiers and has lower toxicity, higherfoaming and stability at extreme pH, temperatures and salinity.[7]

In this study, the exopolysaccharides producing strains *Staphylococcus epidermis and Staphylococcus capiti*s was screened, characterized and various applications like antioxidant activity and emulsifying activity has been traversed.

## MATERIALS AND METHODS

### Screening Oflatic Acid Bacteria

Isolation of exopolysaccharides producing lactic acid bacteria were screened by tenfold serial dilution by spread plate technique. The plates were incubated for 24-48 h at 37°C. The colonies were selected are further streaked on to MRS agar medium. The plates were incubated for 24-48h at 37°C and subculture for further use. The morphological and biochemical characterization of all the isolates have been done. 16s rRNA sequencing were done using primers 16S27F Forward - 5- AGA GTT TGA TCC TGG CTC AG -3' and 16S1492R Universal Reverse – 5'- TAC CTT GTT ACG ACT T -3' and the strains were sequenced and deposited in National Centre for Biotechnology Information (NCBI) database under the Gene Bank accession no. MN736555 for *Staphylococcus epidermis* and MN736556 for *Staphylococcus capitis*. The 16s rRNA gene sequence of the strains was searched in nucleotide sequence database of National Centre for Biotechnology Information (NCBI) by running the BLASTN program.

### Characterization of LAB

### Auto aggregation assay

Overnight cultures were centrifuged at 2000 × g for 10 min at 4°C. Pellets were washed twice in sterile phosphate buffered saline (PBS, pH 7·2) and resuspended in PBS. The suspension (4ml) was mixed by gentle overtaxing for 10s. Absorbance was measured immediately and after 1 h of incubation. The percentage of auto aggregation was expressed as, where At represents the absorbance at 1 h and $A^0$ the absorbance at 0 h. All experiments were performed in triplicates.[8]

$$\text{Auto aggregation (\%)} = 1 - (At/A^0) \times 100$$

### Hydrophobicity assay

Two millilitres of overnight bacterial suspensions were transferred into a fresh tube and 0.4 ml of xylene was added. The tubes were vortexed for 2 min and were allowed to stand for 20 min. The lower aqueous phase was removed and OD at 600 nm was measured. Hydrophobicity (%), was calculated as per the equation Where, $A^0$ and A were the absorbance before and after xylene extraction respectively.

$$\text{Hydrophobicity(\%)} = [(A^0 - A)/A^0] \times 100.$$

### Screening For Exopolysaccharide Production by the Isolated Lab Strains

### Congo Red Agar (CRA) method

Congo red agar medium consist of brain heart infusion 37g/L, sucrose 50g/L, agar powder 10g/L and congo red stain 0.8g/L. Congo red stain was prepared as a concentrated aqueous solution and autoclaved at 121°C for 15 min. The strains were streaked on the CRA plate and incubated aerobically at 37°C for 24 h. Production of black or red colonies with a dry crystalline consistency was considered as positive result.[9]

### Isolation of exopolysaccharide

Isolation was done by growing the exopolysaccharide producing bacterium in 500ml of MRS broth followed by incubation at 37°C for 18-24 hrs. Then14% final volume concentration of trichloroacetic acid was added to denature the protein. The culture broth was incubated at 37°C for 30-40 min in a shaking incubator at 90 rpm. Culture was centrifuged at 8,000 ×g for 20 min to pellet down the cells. The supernatant were mixed with cold absolute ethanol in the ratio of 1:2 (sample supernatant: absolute ethanol). The reaction mixture was incubated at 4°C for 24-48 hrs for precipitation and further centrifuged at 8,000 ×g for 20 min. The pellet was collected and mixed in equal volume of deionized water to each tube containing the precipitate. Lyophilized the precipitate using a freeze drier which is a purified exopolysaccharide.[10]

### Quantification of exopolysaccharides

The isolated exopolysaccharides were resuspended in 1ml of sterile water mixed with 7 mlof 77% Conc. $H_2SO_4$ and transferred to a boiling tube kept in an ice bath for 10 min. 1ml of cold tryptophan was added and the tubes were heated in a boiling bath for 20 min to affect hydrolysis. Acid hydrolysis of EPS produces furan which condenses with the tryptophan to produce

a coloured product. This may be quantified after cooling, by measuring OD at 600nm. Calibration curves were prepared against standard glucose solutions.

### Characterization of exopolysaccharides

Spectroscopic techniques such as UV- visible FTIR were used to evaluate the biophysical properties of the pigment. The surface morphology of black melanin was examined using scanning electron microscopy followed by EDAX (or EDS), an X-ray spectroscopic method for determining elemental compositions like qualitative and quantitative analysis.

### Antioxidant Activity

### DPPH radical scavenging assay

The radical scavenging activity of EPS were done by addition of 500µl of 1.0mM of 1,1- diphenyl-2-hydrazyl(DPPH) in methanolic solution was added to 100 µl of each concentration of exopolysaccharides. DPPH in methanolic solution was used as positive control. After 30 min, the discolouration from deep violet to yellow colour was measured at 515 nm in a spectrophotometer.[11]

### ABTS radical scavenging assay

Solution containing 2-2'-azinobis- 3- ethylbenzothiazoline-6- sulphaonic acid (ABTS) (2.45mM ammonium persulphurfate in 7mM ABTS solution) was kept in dark for 12- 16h at room temperature. Different concentrations of exopolysaccharides were added to 300 µl ABTS solution and the final volume was made upto 1ml using ethanol. After 5 mins the absorbance was read at 745 nm.

### Reduction potential

Different concentrations of sample of 100µl were mixed with 2.5ml of 1% ferricyanide and 2.5ml phosphate buffer (pH 6.6). The mixture was made upto 6ml by using distilled water and incubated at 50°C for 20 min. To 2.5ml of this reaction mixture, equal amount of water was added and 0.5ml of 0.1% ferric chloride was added to it. The absorbance of the colour was measured spectrophotometrically at 700nm.

### Metal chelating activity

The chelation of ferrous irons by the exopolysaccharide pigment was estimated. Different concentration of exopolysaccharide such as 20µl, 50µl and 100µl were mixed with the solution of 2mM $FeCl_2$ (0.05ml). The reaction was initiated by the addition of 5mM ferrozine (0.2ml) and the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562nm. All the tests and analysis were done in duplicates and averaged.[12] The inhibition of exopolysaccharides, metal chelating activity in percentage was calculated by the following equation

$$\text{Metal chelating effect (\%)} = [(A_O - A_1)/A_O] \times 100$$

Where,

$A_O$ is the absorbance of the control reaction

$A_1$ is the absorbance in the presence of sample

The control used to contain $FeCl_2$ and ferrozine.

### Determination of Emulsification Activity of EPS

Various hydrocarbons and vegetable oils were used. Hydrocarbon or vegetable oil (1.5 mL) was added to 1.5 mL of various concentrations of EPS (0.5, 1.0 and 1.5 mg/mL) and vortexed vigorously for 2 min. The emulsification activity (% EA) was determined after 1 hr whereas the emulsion stability was determined as emulsification index (% EI) after 24, 48 and 72 hrs. The % EA and % EI were calculated by dividing the height of the emulsion layer (in cms) by the total height of the mixture (in cm) multiplied by 100.[13]

## RESULTS

### Screening of LAB

Lactic acid bacteria was isolated from goat gut, serially diluted in MRS broth and plated in MRS agar by spread plate technique. The colonies of bacteria were observed and selected. The lactic acid bacteria producers were characterized phenotypically and genotypically to identify them. Gram staining of Exopolysaccharides producing isolates showed Gram negative cocci. The catalase showed positive which indicates the presence of enzyme catalase. Coagulase test showed negative indicating the absence of the enzyme coagulase produced by *Staphylococcus aureus*. Biochemical characterization revealed that it may belongs to the same genus (Table 1). The 16s rRNA gene were amplified using specific primers and amplified products were visualized in agarose gel electrophoresis. The amplicons were sequenced using 16s rDNA sequencing and the identity of the sequence were determined using NCBI blast where the sequence was searched against GenBank database. The obtained sequences were further submitted to obtain accession numbers.

**Table 1: Biochemical characteristics of isolated culture.**

| Characterization | *Staphylococcus epidermis* | *Staphylococcus capitis* |
|---|---|---|
| Gram staining | + | + |
| Coagulase test | - | - |
| Catalase test | + | + |

**Table 2: Percentage of Auto Aggregation Assay.**

| S.No | Strains | Auto aggregation (%) |
|---|---|---|
| 1 | *Staphylococcus epidermis* | 21.3% |
| 2 | *Staphylococcuscapitis* | 58.2% |

**Table 3: Hydrophobicity Assay values in percentage.**

| S.No | Strains | Hydrophobicity(%) |
|---|---|---|
| 1 | *Staphylococcus epidermis* | 71.8% |
| 2 | *Staphylococcuscapitis* | 64% |

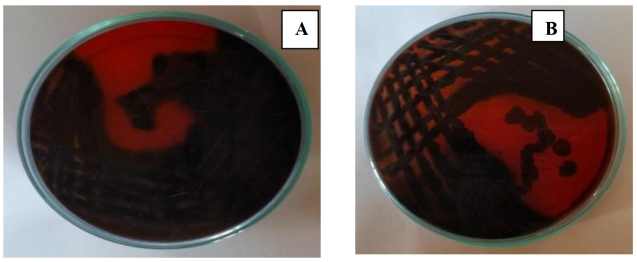


**Figure 1: Colonies of isolated strains producing exopolysaccharide on Congo red agar with 50 g/l of sucrose. A - black or almost black colonies (positive), B - black or almost black colonies (positive).**

## Auto aggregation assay

The sedimentation rate of the cultures was measured after 1 h. Results indicated that the strains exhibited a strong auto aggregation as mentioned below in Table 2.

## Hydrophobicity Assay

The hydrophobicity analysis showed that isolates were strongly hydrophobic with adhesion to xylene as mentioned below in Table 3

## Identification of exopolysaccharides

## Congo red agar (CRA) method

The synthesis of EPS was determined by growing the cultures in congo red agar and production of black colonies by the isolates *Staphylococcus epidermis* and *Staphylococcus capitis* showed that they have the ability to synthesize Exopolysaccharides. (Figure 1)

## Isolation of exopolysaccharides

EPS was isolated, extracted and purified from both the culture. Further, EPS was stored as a stock and used for both qualitative and quantitative studies.

## Quantification of exopolysaccharides

The quantitative assay for EPS was done and calibration curves were prepared against standard glucose solutions and results were found to be 93µg/ml for *Staphylococcus epidermis* and 135 µg/ml for *Staphylococcus capitis*.

## Characterization of exopolysaccharides

FTIR analysis was used to identify the molecules, proteins and functional groups (Table 4 and Table 5). The FTIR analysis of EPS of *Staphylococcus epidermis* showed the absorption peaks located at 3738.05cm$^{-1}$ (Alcohol O-H stretch) 2304.94 cm$^{-1}$ (Aromatic compound C-H bonding) 1514.12 cm$^{-1}$ (Nitro compound N-O stretch) and 680.87 cm$^{-1}$ (Halo compound C-BR stretch) and for the EPS of *Staphylococcus capitis* the absorption peak were located at 3400 cm$^{-1}$ (Aliphatic primary amine N-H stretch) 2304.94 cm$^{-1}$ (Carbon dioxide O=C=O stretch) 1645.28 cm$^{-1}$ (Conjugated alkene C=C stretch) 1516.05 cm$^{-1}$ (Nitro compound N-O stretch) 1394.53 cm$^{-1}$ (Alcohol O-H bonding) 1058.92 cm$^{-1}$ (Primary alcohol C-O stretch) 599.86 cm$^{-1}$ (Halo compounds C-I stretch). SEM analysis was carried out to determine the morphological structures of the EPS. The ultra-structure of

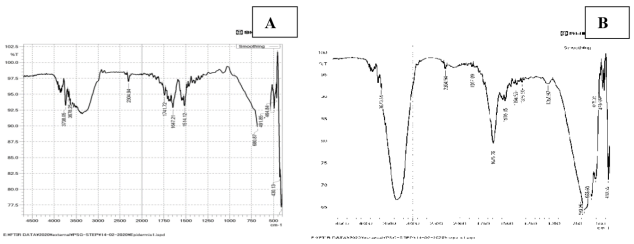


**Figure 2: FTIR analysis of EPS synthesized from *Staphylococcus epidermis* (A) and *Staphylococcus capitis* (B).**

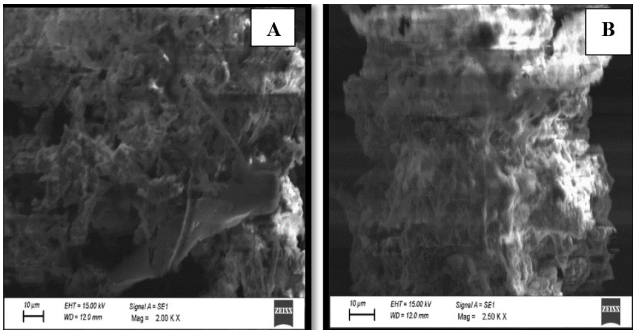


**Figure 3: A and B -Scanning electron micrograph of EPS synthesised from *Staphylococcus epidermis*.**

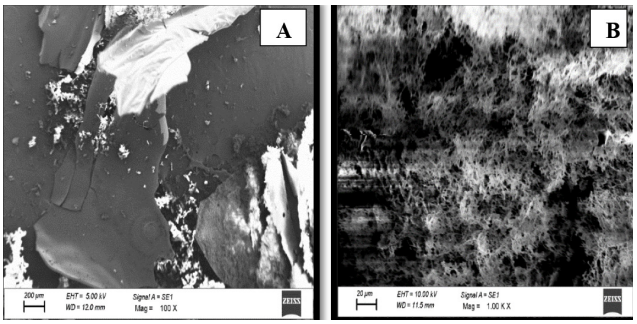


**Figure 4: A and B -Scanning electron micrograph of EPS synthesised from *Staphylococcus capitis*.**

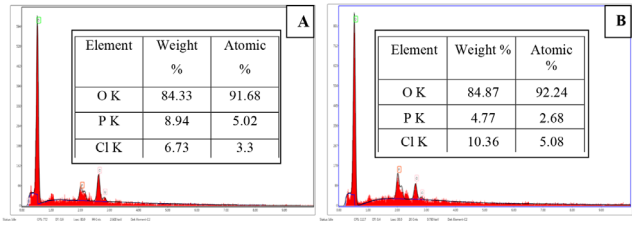


**Figure 5: The Table (A) (B) shows the elemental composition of the EPS along with their weight % and atomic % of *Staphylococcus epidermis* and *Staphylococcus capitis*.**

**Table 4: Functional groups recorded from EPS synthesized from *Staphylococcus epidermis*.**

| S.No | Wave number ($cm^{-1}$) | Functional Group |
|---|---|---|
| 1 | 3738.05 | Alcohol O-H stretch |
| 2 | 2304.94 | Aromatic compound C-H bonding |
| 3 | 1514.12 | Nitro compound N-O stretch |
| 4 | 680.87 | Halo compound C-BR strech |

**Table 5: Functional groups recorded from EPS synthesized from *Staphylococcus capitis*.**

| S.No | Wave number ($cm^{-1}$) | Functional Group |
|---|---|---|
| 1 | 3400 | Aliphatic primaryamine N-H stretch |
| 2 | 2304.94 | Carbon dioxide O=C=O stretch |
| 3 | 1645.28 | Conjugated alkene C=C stretch |
| 4 | 1516.05 | Nitro compound N-O stretch |
| 5 | 1394.53 | Alcohol O-H bonding |
| 6 | 1058.92 | Primary alcohol C-O stretch |
| 7 | 599.86 | Halo compounds C-I stretch |

both the EPS were observed under various magnification (Figure 3,4).

The elemental compositional study was observed in both EPS of *Staphylococcus epidermis* and *Staphylococcus capitis*. The Table (a) (b) shows the elemental composition

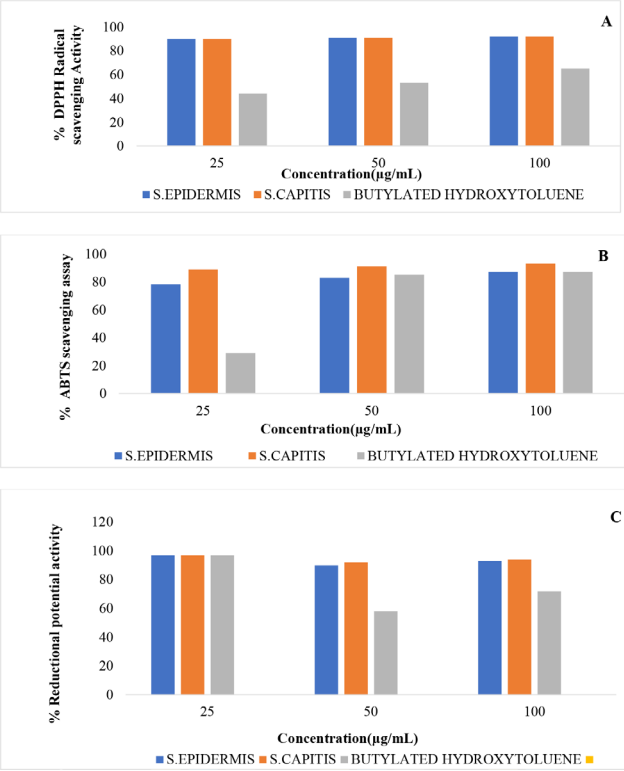


**Figure 6: A, B, C denotes DPPH radical scavenging, ABTS radical scavenging and reduction potential respectively.**

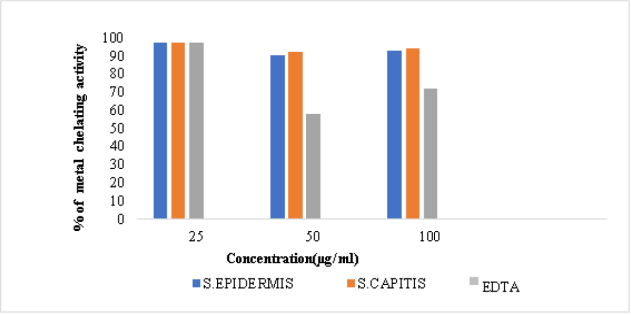


**Figure 7: Metal chelating activity.**

of the EPS along with their weight % and atomic % of *Staphylococcus epidermis* and *Staphylococcus capitis* (Figure 5).

## Antioxidant activity

Antioxidant activity was done to check the free radical scavenging ability of the EPS. The results were observed that high inhibition value of free radicals with increasing concentration of EPS. (Figure 6)

## Metal chelating activity

Metal chelating was done to check the chelation of metal ions. Ferrozine can quantitatively form complexes with $Fe^{2+}$. Both purified EPS showed metal chelating activity. (Figure 7)

### Determination of Emulsification Activity (EA)

An emulsion was prepared with different vegetable oils (sunflower oil and coconut oil) and hydrocarbons (petrol, diesel and xylene). The EPS of *Staphylococcus epidermis* and *Staphylococcus capitis* was capable of stabilizing emulsions with different oils and hydrocarbons. For *Staphylococcus epidermis,* the index values were higher for coconut oil (62.5%) Both EA and EI for coconut oil were higher when compared with those of other solvents. For *Staphylococcus capitis,* the index values were higher for coconut oil (61.5%). Both EA and EI for coconut oil were higher when compared with those of other solvents. Hence EPS produced by *Staphylococcus epidermis* and *Staphylococcus capitis* can be used as emulsifier in foods, especially where coconut oil is used. (Table 6 and Table 7)

**Table 6: Emulsifying activity (EA) of exopolysaccharide of *Staphylococcus epidermis* with different concentrations under different vegetable oils and hydrocarbons.**

| Hydrocarbon/ oil | EPS mg/ml | % EA | Emulsifying index | | |
|---|---|---|---|---|---|
| | | | $E_{24}$ | $E_{48}$ | $E_{72}$ |
| Petrol | 0.5 | 45 | 50.5 | 52.5 | 53.5 |
| | 1 | 47.5 | 52.1 | 56.2 | 56.5 |
| | 1.5 | 55 | 57.5 | 59.5 | 59.5 |
| Diesel | 0.5 | 40 | 44.5 | 49.5 | 52.5 |
| | 1 | 47.5 | 50.5 | 54.5 | 59.5 |
| | 1.5 | 49 | 52.5 | 59.5 | 60.5 |
| Coconut oil | 0.5 | 42.5 | 45.5 | 49.5 | 52.5 |
| | 1 | 44.5 | 49.5 | 53.5 | 56.5 |
| | 1.5 | 47.5 | 51.5 | 55.5 | 62.5 |
| Palm oil | 0.5 | 32.5 | 36.5 | 42.5 | 44.5 |
| | 1 | 36.5 | 39.5 | 44.5 | 46.5 |
| | 1.5 | 38.5 | 41.5 | 49.5 | 51.5 |
| Xylene | 0.5 | 42.5 | 45.5 | 49.5 | 53.5 |
| | 1 | 44.5 | 48.5 | 52.5 | 55.5 |
| | 1.5 | 49.5 | 51.5 | 55.5 | 59.5 |

**Table 7: Emulsifying activity (EA) of exopolysaccharide of *Staphylococcus capitis* with different concentrations under different vegetable oils and hydrocarbons.**

| Hydrocarbon/ oil | EPS mg/ml | % EA | Emulsifying index | | |
|---|---|---|---|---|---|
| | | | $E_{24}$ | $E_{48}$ | $E_{72}$ |
| Petrol | 0.5 | 39.5 | 45.5 | 51.5 | 57.5 |
| | 1 | 42.5 | 49.5 | 58.2 | 60.5 |
| | 1.5 | 44.5 | 51.5 | 59.5 | 59.5 |
| Diesel | 0.5 | 32.5 | 39.5 | 45.5 | 51.5 |
| | 1 | 34.5 | 40.5 | 47.5 | 49.5 |
| | 1.5 | 37.5 | 44.5 | 52.5 | 60.5 |
| Coconut oil | 0.5 | 39.5 | 45.5 | 51.5 | 57.5 |
| | 1 | 41.5 | 47.5 | 53.5 | 52.5 |
| | 1.5 | 45.5 | 53.5 | 59.5 | 61.5 |
| Palm oil | 0.5 | 36.5 | 40.5 | 48.5 | 51.5 |
| | 1 | 39.5 | 47.5 | 52.5 | 55.5 |
| | 1.5 | 44.5 | 49.5 | 52.5 | 53.5 |
| Xylene | 0.5 | 41.5 | 46.5 | 52.5 | 56.5 |
| | 1 | 45.5 | 48.5 | 55.5 | 58.5 |
| | 1.5 | 47.5 | 51.5 | 57.5 | 60.5 |

## DISCUSSION

In the present study, the lactic acid bacteria were isolated from the goat gut and were characterized phenotypically and genotypic ally to identify them. Gram staining of Exopolysaccharides producing isolates showed Gram negative cocci. The catalase showed positive which indicates the presence of enzyme catalase. Coagulase test showed negative indicating the absence of the enzyme coagulase that is produced by *Staphylococcus aureus*. Biochemical characterization revealed that it may belong to the same genus. The isolates producing lactic acid bacteria was identified upto species level by 16S rDNA sequence analysis and found to be *Staphylococcus epidermis* and *Staphylococcus capitis*. Altschul *et al.* (1990) reported that 16S rRNA gene were amplified using specific primers and amplified products were visualized by agarose gel electrophoresis. The amplicons were sequenced using 16S rDNA sequencing and the identity of the sequences was determined using NCBI blast where the sequences were searched against Gen Bank database. The obtained sequences were further submitted to obtain accession numbers. The sedimentation rate and hydrophobicity were measured and the results show that the strains exhibited strong Auto aggregation and hydrophobic adhesion to xylene. Synthesis of Exopolysaccharide was determined by growing the cultures in Congo Red agar and visual identification of black colonies proved to have EPS production, namely *Staphylococcus epidermis* and *Staphylococcus capitis* had good ability to synthesize EPS. Further EPS was isolated, extracted and purified from the cultures. This was followed by quantification of EPS against standard glucose solution and the results were found to be 93 µg/ml for *Staphylococcus epidermis* and 135 µg/ml for *Staphylococcus capitis*. Both the isolates *Staphylococcus capitis* and *Staphylococcus epidermis* showed to have potential radical scavenging activity comparing with the standard Butylated hydroxytoluene. The isolates showed increasing inhibition with increase in concentration. Ferrozine can quantitatively form complexes with Fe2+. Both EPS shows metal chelating activity. EPS producing strain

*Staphylococcus epidermis* and *Staphylococcus capitis* exhibited good emulsification activity. An emulsion was prepared with different vegetable oils (sunflower oil and coconut oil) and hydrocarbons (petrol, diesel and xylene). The EPS of *Staphylococcus epidermis and Staphylococcus capitis* was capable of stabilizing emulsions with different oils and hydrocarbons. For *Staphylococcus epidermis* the index values were higher for coconut oil (62.5%) both EA and EI for coconut oil were higher when compared with those of other solvents. For *Staphylococcus capitis* the index values were higher for coconut oil (61.5%) both EA and EI for coconut oil were higher when compared with those of other solvents. Hence the EPS produced by *Staphylococcus epidermis and Staphylococcus capitis* can be used as emulsifier in foods, especially where coconut oil is used.

## CONCLUSION

Lactic acid bacteria, being an EPS producer, the isolates *Staphylococcus epidermis* and *Staphylococcus capitis* can be employed in the production of fermented foods like yoghurt. Its intense bioactivities such as antioxidant, metal chelating and emulsification, tend to be used as a potent bio emulsifier in food industries.

## ACKNOWLEDGEMENT

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## CONFLICT OF INTEREST

No conflict of interest was declared.

## ABBREVIATIONS

**EPS:** Exopolysaccharide; **LAB:** Lactic Acid Bacteria; **CRS:** Congo Red Agar; **SEM:** Scanning Electron Microscope; **FTIR:** Fourier Transform Infrared Spectroscopy; **EDAX:** Energy Dispersive X ray analysis; **PCR:** Polymerase Chain Reaction; **BLASTn**: Basic Local Alignment Search Tools nucleotide; **EA:** Emulsifying activity.

## SUMMARY

Being an EPS producer, the isolates *Staphylococcus epidermis* and *Staphylococcus capitis* may be employed in the production of fermented foods like yoghurt. Their radical scavenging activities indicate pharmacological applications and inclusion in functional foods. The purified EPS of the isolates can be potential emulsifier.

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