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IN VITRO ANTIOXIDANT EFFICACY OF EPS OBTAINED FROM *MICROCOCCUS LUTEUS* SNIST- CM 02: A BRIEF STUDY

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ABSTRACT

The polysaccharides biosynthesized by the microorganisms had emerged as an important class of bioactive compound from the last two decades. In the present study, an attempt was taken to evaluate the *in vitro* antioxidant activity of EPS produced by *Micrococcus luteus* SNIST-CM02. The scavenging assays involving 1, 1-diphenyl-2-picrylhydrazyl (DPPH), superoxide radicals, hydroxyl radicals, chelating ability and inhibition effect of lipid peroxidation, for the biosynthesized EPS were studied in order to evaluate the *in vitro* antioxidant activity. The EC50 value of EPS for DPPH, superoxide radicals and hydroxyl radicals was found to be 8.11 mg/mL, 8.00 mg/mL and 10.25 mg/mL respectively. The EPS had a lower inhibition effect of lipid peroxidation than that of ascorbic acid and butylated hydroxytoluene. At a concentration of 12.0 mg/mL, the inhibition was only 70.00%. These results suggest that the EPS synthesized by *Micrococcus luteus* SNIST- CM02 can be used as a good potential antioxidant and can be added to different types of food as a novel antioxidant.

Keywords: Antioxidant; DPPH; Scavenging assay; *Micrococcus luteus*; EPS; EC_{50} Value

INTRODUCTION

The free radicals cause the harmful effect to living system either by the onset of degenerative diseases or by causing the ageing phenomena very fast **(Cross *et al.*, 1987; Beckman and Ames 1998).** Generally, these phenomena are known as oxidative stress **(Michiels *et al.*, 1994)**. There are so many compounds which present in the living cell, significantly cease this oxidative stress and these all are collectively known as antioxidant. Although, the cells have its own mechanism to protect itself from the stress, but in some cases the indigenous or exogenous sources saddle of the free radicals lead to the imbalance the redox potential of the living cell. In order to overcome this, the cells need the other alternative antioxidant from outside as a supplement. Therefore, naturally occurring antioxidant molecules have drawn a great attention in the field of medicine and food. Recently, the polysaccharide produced from the microorganism has been explored as the novel antioxidant **(Michiels *et al.*, 1994; Tiwari 2001).**
Generally, EPS synthesized by microorganisms are non-toxic, non-immunogenic, non-carcinogenic and biocompatible in nature. Different types of application related to the exopolymers are like food packaging, capsule coating, gene delivery, drug delivery, molecular chaperon synthesis have been carried out by various authors **(Duan *et al.*, 2007; Gheorghe *et al.*, 2008; Yoshihiro *et al.*, 2010).**
The EPS is biosynthesised in the cytoplasm and then secreted into the extracellular environment facilitating its recovery **(Li *et al.*, 2006)**. The biosynthetic pathways and its real mechanism of synthesis of various EPSs are not clearly understood. Few years back, some authors **(Duan *et al.*, 2007)** have proposed a biosynthesis pathway model for the EPS synthesis. The exopolysaccharide bio-synthesized by microorganisms, including bacteria, moulds and yeast, represent an unexploited market **(Sutherland, 2001)**. But at the same time, the limiting factor of EPS production by microorganisms is linked to its production cost. The main costs consist of the price of the carbon and nitrogen sources in certain cases **(Donot *et al.*, 2012).**
In the present study, the antioxidant activity of the crude exopolysaccharide (EPS) from a newly isolated strain of *Micrococcus luteus* SNIST- CM02 (screened from the natural habitat) was studied. Carbohydrate based antioxidants are having better applicability over other antioxidants as they are neutral, water soluble and non-toxic to the living cells.

MATERIALS AND METHODS

Microbial strain, culture conditions and eps production

The production and characterization of EPS was performed in our previous study **(Mishra *et al.*, 2016)** from the isolate of *Micrococcus luteus* SNIST- CM02. In this process, EPS was produced in the 250mL Erlenmeyer flask. The composition of the medium for the production of EPS was as follows: Sucrose 60.0 g, K_2HPO_4 7.5 g, NaCl 1.5 g, $MgSO_4.7H_2O$ 0.4 g, Yeast Extract 0.4 g and distilled water 1,000 mL. The pH of the medium was adjusted to 6.5 by adding 0.1 M NaOH. After preparation and sterilization of the medium, 1 mL of the prepared inoculum was inoculated to 100 mL of the production medium and was incubated for 6 days under agitation (150 rpm) at 25°C. The microorganism was maintained on Nutrient agar at 4°C and sub-cultured every 2 weeks. The isolated strain was inoculated to the production medium (Sucrose 60.0 g, K2HPO4 7.5 g, NaCl 1.5 g, MgSO4.7H2O 0.4 g, Yeast Extract 0.4 g and distilled water 1000 mL) and was incubated for 7 days under agitation (150 rpm) at 28 °C. The recovery process for the EPS production was carried out first by removing the cells by centrifugation at 7,000 rpm, for 10 min followed by its precipitation with adding twice the volume of cold isopropyl alcohol **(Singh *et al.*, 2009).**

Preparation of exopolysaccharide (EPS)

Crude EPS was purified by dissolving the precipitates with the water. Further, the solution of the EPS was reprecipitated with ice-chilled isopropyl alcohol. This methodology was followed up to attainment of the 3rd fraction. Each fraction of EPS obtained after the precipitation was made to wash with the acetone before reprecipitation. The pure EPS obtained in 3rd fraction was further subjected to dialysis in order to analyze the *in-vitro* antioxidant activities.

IN VITRO ANALYSIS OF ANTI-OXIDANT ACTIVITY OF EPS

Scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals

The antioxidant analysis was carried out by using 1 mL of EPS solutions was made at different concentrations (0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0 and 16.0 mg/mL) and was added to 4.0 mL of 0.004% solution of DPPH in ethanol. After 30 min, the absorbance was measured at 517 nm. Scavenging ability (%) of DPPH radicals = $\{1- (A_{sample} / A_{control})\} \times 100$, where $A_{control}$ is the absorbance of control, and A_{sample} is the absorbance in the presence of the tested samples. The ascorbic acid (Vitamin C) and butylated hydroxytoluene were taken as positive controls for this study **(Shimada *et al.*, 1992; Guo *et al.*, 2010).**

Scavenging of superoxide radicals

Antioxidant analysis was further analysed by adding, 4 mL of Tris–HCl buffer (pH 8.2) and 1 mL of EPS sample solution with different ranges of concentrations (0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0 and 16.0 mg/mL) were incubated at 30 °C for15 min, followed by the addition of 200 µL of pyrogallol to the mixture, and the reaction was allowed to proceed for 4 min. The reaction was made to stop by adding 0.5 mL of HCl. The absorbance of the mixture was measured at 320 nm. Scavenging ability (%) of superoxide radicals = {1- (A_{sample} / $A_{control}$)} ×100, where $A_{control}$ is the absorbance of control, and A_{sample} is the absorbance in the presence of the tested samples. The ascorbic acid (Vitamin C) and butylated hydroxytoluene were taken as positive controls for this study **(Marklund and Marklund, 1974).**

Hydroxyl radical scavenging activity

The reaction mixture containing EPS (0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0 and 16.0 mg/mL) was incubated with deoxyribose (3.75 mM), H_2O_2 (1 mM), $FeCl_3$ (100 mM), EDTA (100 mM) and ascorbic acid (100 mM) in potassium phosphate buffer (20 mM, pH 7.4) for 60 min at 37 °C **(Halliwell *et al*., 1987).** The reaction was terminated by adding 1 mL of thio-barbituric acid (1%, w/v) and 1 mL of trichloroacetic acid (2%, w/v) following the incubation in a boiling water bath for 15 min. The absorbance of the mixture was measured at 535 nm against reagent blank. The Scavenging ability (%) of hydroxyl radicals = {1- (A_{sample} / $A_{control}$)} ×100, where $A_{control}$ is the absorbance of control, and A_{sample} is the absorbance in the presence of the tested samples. The ascorbic acid (Vitamin C) and butylated hydroxytoluene were taken as positive controls for this study.

Chelating ability of eps on ferrous ion

The chelating ability of EPS with the ferrous ion (Fe^{2+}) was studied according to the protocol given by **Decker and Welch (1990)**. The Fe^{2+} chelating ability of EPS from *Micrococcus luteus* SNIST- CM02 was studied by measuring the FerroZine iron complex at 562 nm. Here the EDTA was used as a positive control.

Inhibition effect of lipid peroxidation

The antioxidant analysis was finally confirmed by the standard method as given by Kimuya et al. (1981) for effect of lipid peroxidation was followed in which 0.8 mL of egg yolk mixed with 0.1 mol/L phosphate buffer (pH 7.45) and resulted homogenate was added to 0.5 mL of EPS sample solution with different concentrations (0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0 and 16.0 mg/mL). In order to initiate the lipid peroxidation process, 0.5 mL of 25 mmol/L of $FeCl_2$ was added to the above mixture. After incubation at 37°C for 1 hour, 1.0 mL of 20% (w/v) trichloroacetic acid and 1.0 mL of 0.8% (w/v) thiobarbituric acid were added to stop the reaction. The mixture was heated at 100 °C for 20 min, and centrifuged (2400g) for 10 min. The upper layer was collected, and the absorbance was taken at 532 nm. The inhibition (%) effect= {1- (A_{sample} / $A_{control}$)} ×100 was calculated, in which $A_{control}$ is the absorbance of control and A_{sample} is the absorbance in the presence of the tested samples. The Ascorbic acid (Vitamin C) and Butylated hydroxytoluene were taken as positive controls for this study.

RESULTS

***In vitro* anti-oxidant activity of EPS**

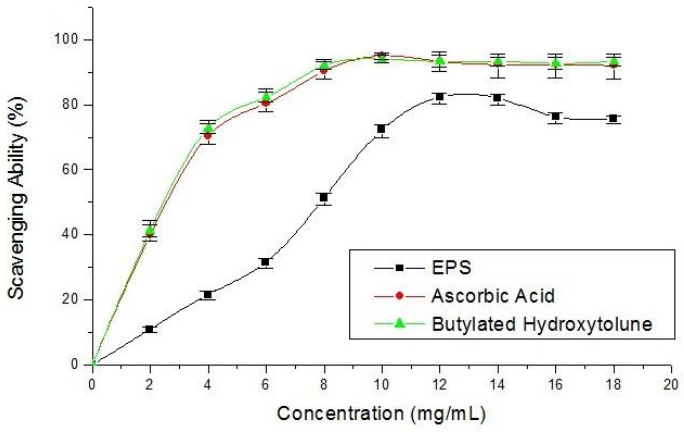

Figure 1 Scavenging of DPPH radicals (Values were representative of three separated experiments)

The *in-vitro* antioxidant property of the EPS had been assessed with DPPH scavenging, superoxide radicals scavenging and lipid peroxidation inhibition assays. These antioxidant activities were compared with those of ascorbic acid and butylated hydroxytoluene. Less scavenging of DPPH and superoxide radicals was observed with EPS than with ascorbic acid and butylated hydroxytoluene at concentration < 12.0 mg/mL, but at the same time the scavenging ability was similar to those of ascorbic acid and butylated hydroxytoluene at concentration ≥ 12.0 mg/mL as found in figure 1 and figure 2.

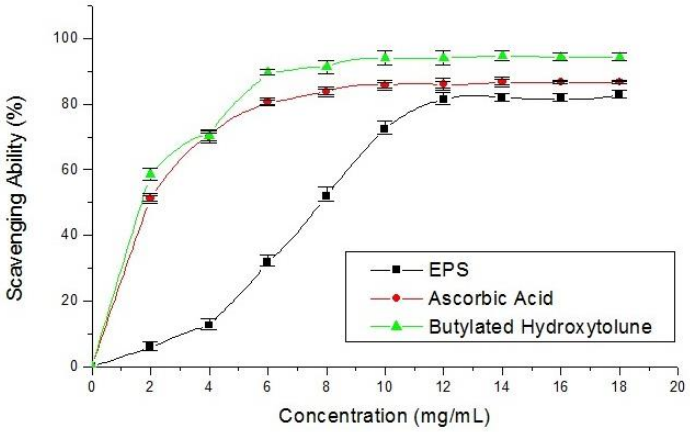

Figure 2 Scavenging of superoxide radicals (Values were representative of three separated experiments)

The scavenging efficacy of the hydroxyl radicals for EPS was found to be same with ascorbic acid and butylated hydroxytoluene at concentration ≥ 8.0 mg/mL. It was observed that, the scavenging ability of EPS sharply increased when the concentration changed from 8.0 mg/mL to 12.0 mg/mL as shown in figure 3.

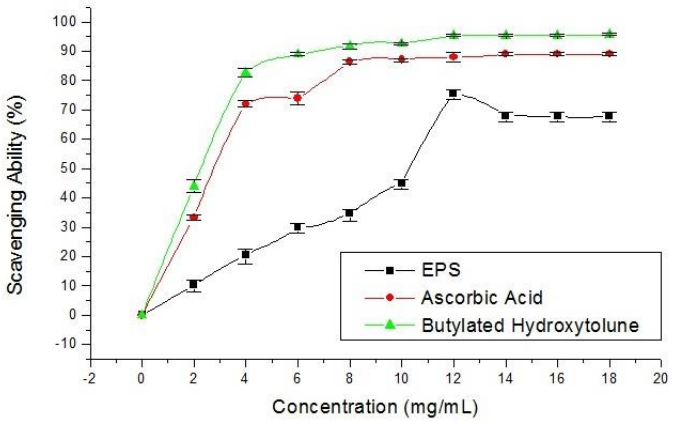

Figure 3 Scavenging of hydroxyl radicals (Values were representative of three separated experiments)

The chelating activity for EPS was also estimated and it was found nearly equal to 70 % at concentration ≥ 6.0 mg/mL of EPS as shown in figure 4. The chelating ability of EPS was lesser than that of EDTA. Previously the chelating activity of some of the carbohydrates like sulfated polysaccharides, acetylated polysaccharides and phosphorylated polysaccharides from *Ramulus mori* was found to be 74.5%, 67.2% and 58.7% at 2.1 mg/mL, respectively **(Zhang *et al.*, 2008).**

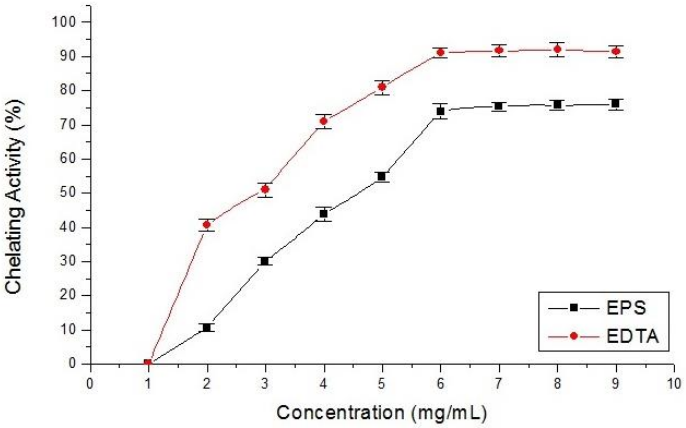

Figure 4 Chelating ability of EPS with ferrous ion (Values were representative of three separated experiments

The EC_{50} value of EPS for DPPH radical is 8.11 mg/mL and for superoxide radical it is 8.0 mg/mL. For the hydroxyl radicals, the EC_{50} value was found to be

10.25 mg/mL. The EPS had a lower inhibition effect of lipid peroxidation than that of ascorbic acid and butylated hydroxytoluene. At the concentration of 12.0 mg/mL, the inhibition was 70.0% as shown in figure 5. These results suggest that the EPS synthesized by *Micrococcus luteus* SNIST- CM02 can be used as a good potential antioxidant.

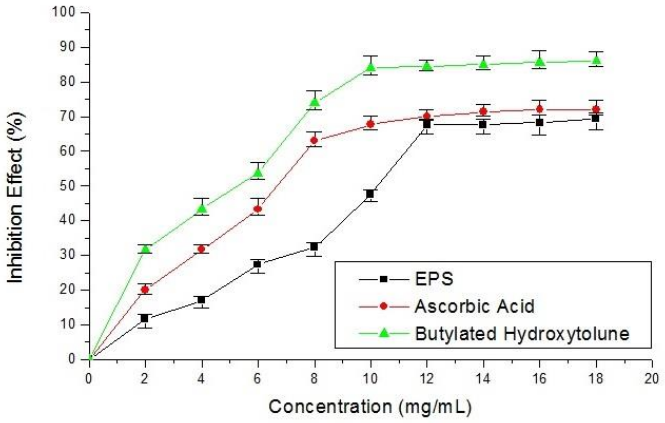

Figure 5 Lipid peroxidation Inhibition (Values were representative of three separated experiments)

DISCUSSION

The results of the present findings depict that EPS from *Micrococcus luteus* SNIST- CM02 have good antioxidant and antiradical activities. From the different types of experiments, it was found that EPS exhibited the concentration dependent antioxidant activity. The antioxidant activity of the microbial EPS probably related to the monosaccharide component, molecular size, conformation and due to its bioactivity. It has been reported that the bioactivities of polysaccharides are closely associated with the type of glycosyl units, the configuration of glycosidic bonds, and the substituents of the polysaccharides. Additionally, the spatial structure and relative molecular mass of polysaccharides conjointly have an effect on the bioactivity **(Tsiapali *et al.*, 2001).** Therefore, the antioxidative activity of the EPS is not the results of any single factor. It is the results of several factors combined within the variation of carbohydrate composition, structural configuration, and mode of attending glycosidic bonds, molecular weight and other structural characteristics. Moreover, the monosaccharides in the EPS can be acted as reductive agents and these can add hydrogen, which when combined with radical forms a more stable radical to terminate the free radical chain reaction.

The model of scavenging the DPPH radical is a widely to evaluate the free radical-scavenging activities of polysaccharides **(Soares *et al.*, 1997; Naik *et al.*, 2003)**. The method is based upon the decrease in the absorbance of methanolic solution of DPPH at 517 nm in the presence of EPS which acts as a proton-donor as shown in figure 1. EPS may be reacting with DPPH radicals to convert them to more stable products and thereby terminate radical chain reactions.

The superoxide radicals are generally formed first in the cellular oxidation reactions and its effect can be a precursor for the generation of other types of cell-damaging free radicals **(Liu and Ng, 2000).** The superoxide radical was generated by the autooxidation of pyrogallol and it can form a coloured compound resulting purple to yellow colour. The absorbance at 320 nm increased when the superoxide radical was scavenged by an antioxidant. The increment in the absorbance represent the content of superoxide anions and also indicate the antioxidant activity of the sample **(Chen *et al.*, 2008).** The results suggested that the EPS can be used as effective scavenger for superoxide radicals (figure 2), and it might be advantageous for preventing injury caused by superoxide radicals in adverse pathological conditions.

Hydroxyl radicals are known as highly potent oxidant, which can react with most of the biomacromolecules in living cells and induce severe catabolic activities to the adjacent biomolecules. Therefore, it is important for removing hydroxyl radical for antioxidant defence in cell or food systems. The present results proved that the EPS isolated from *Micrococcus luteus* SNIST- CM02 was a good scavenger for hydroxyl radicals as shown in figure 3.

Chelating agents may serve as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ions. It was concluded that the microbial EPS could be used as a novel chelating agent from this studies. The chelating ability was comparable with the EDTA as shown in figure 4.

The lipid peroxidation is a process, in which the free radicals steal the electron from the lipids of the cell membrane. This results in cell damage. This is generally occurred in various pathological events like inflammation and cellular aging **(Wiseman and Halliwell, 1996)**. In this study, a yolk of egg suspension was used to evaluate the inhibitory activities of lipid peroxidation with EPS. The egg yolk lipids undergo rapid peroxidation when incubated with $FeCl_2$. The inhibiting effects of the EPS on the lipid peroxidation was found to be a concentration dependent process as shown in figure 5.

CONCLUSION

In this paper, we have studied the antioxidant aspect like scavenging of DPPH radicals, scavenging of super oxide radical, scavenging of hydroxyl radical, chelating of metal ion and lipid peroxidation inhibition studies of the EPS synthesized by *Micrococcus luteus* SNIST- CM02. The present investigation suggested that the EPS isolated from *Micrococcus luteus* SNIST-CM02 could be helpful and beneficial to alleviate the oxidative damages in cell induced by oxygen radicals and decelerate the progress of many chronic diseases in human body. However, the *in vivo* antioxidant activity and the antioxidant mechanism of EPS need to be analyzed in future.

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