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EXTRACTION, PARTIAL CHARACTERIZATION AND ANTIBACTERIAL EFFICACY OF EXTRA CELLULAR POLYSACCHARIDES FROM BACILLUS LICHENIFORMIS AND KLEBSIELLA PNEUMONIAE ISOLATED FROM ROOT NODULE OF TEPHROSIA PURPUREA

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ABSTRACT

Many bacteria produce extra cellular polysaccharide (EPS) and in genera such as *Klebsiella, Rhizobium, Bacillus* and *Streptococcus* they[Delete] have been well characterized for a number of species and strains. Polysaccharides from plant sources material and their modified forms are important additives in the food industry. The EPS is the use of xanthan in oil industry. The most significant biomedical exploitation of bacterial polysaccharides is used as vaccine agent. In the present study, the authors describe the selection of the plant *Tephrosia purpurea* was selected[delete], since it contains many species producing EPS. From the root nodule of this plant, five different species of bacteria were isolated and studied for their antibacterial activity. Out of five strains, two strains showed promising activity against human pathogens (*Escherichia coli, Enterococcus aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus*) and these two strains were used for further studies. Molecular characterization for best strains was done and found to be *Bacillus licheniformis* and *Klebsiella pneumonia*. The growth curves of *B.licheniformis* and *K. pneumoniae* were studied. Also EPS were isolated and estimated by phenol sulphuric acid method in UV-Vis spectrophotometer. The large amounts of EPS 210 mg/ml from *K.pneumoniae* and 70.02 mg/ml from *B.licheniformis* were estimated before dialysis and after dialysis 12.3 mg/ml from *K.pneumoniae* and 10.97 mg/ml from *B.licheniformis* were estimated. Three polysaccharides components were observed by HPLC analysis. Also lyophilization was being used for the production of extra cellular polysaccharides.

Key words: **EPS, *Tephrosia purpurea*, *Bacillus licheniformis*, *Klebsiella pneumonia*.**

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INTRODUCTION

Tephrosia purpurea is a species of flowering plant in the pea family, fabacea that has a pantropical distribution. It is a common wasteland weed. In many parts it is under cultivation as green manure crop. It is found throughout India and Sri Lanka in poor soils (Arnold, *et al.,* 1968). The root and seed are reported to have insecticidal and piscicidal properties and also used as vermifuge (Zafar, *et al.,* 2004). The roots are also reported to be effective in leprous wound and their juice is applied to the eruption on skin. Its aerial parts and roots are used in bronchial asthma, hepatic ailments, pain and inflammation. *Tephrosia purpurea* is a wild plant known as “Sarapunkha” in Sanskrit,‘Purple tephrosia’ or ‘Wild indigo’ in English and “Avuri” or “Kolinji” in Tamil and 'Unhali' in Gujarati. In Ayurvedic system of medicine various part of this plant are used as remedy for impotency, asthma, diarrhea, gonorrhea, ulcear and urinary disorders (Joshi 2000).Root nodules occur on the roots of plants that associate with symbiotic nitrogen-fixing bacteria. Under nitrogen limiting conditions, capable plants form a symbiotic relationship with a host-specific strain of bacteria known as rhizobia. This process has evolved multiple times within the Fabaceae, as well as in other species found within the Rosid clade (Doyle, *et al.,* 2003). With the legume nodules, nitrogen gas from the atmosphere is converted into ammonia, which is then assimilated into amino acids, nucleotides (the building blocks of DNA and RNA as well as the important energy molecule ATP), and other cellular constituents such as vitamins, flavones, and hormones. Nitrogen fixation in the nodule is very oxygen sensitive. Legume nodules iron containing protein called leghaemoglobin, closely related to animal myoglobin, to facilitate the conversion of nitrogen gas to ammonia.

Many bacteria produce extra cellular polysaccharide (EPS) and in genera such as *Bacillus, Klebsiella, Rhizobium* and *Streptococcus* they have been well characterized for a number of species and strains. Bacteria within the genera *Agrobacterium* and *Rhizobium* have the unique capacity to produce more amounts of polysaccharides and induce prolific root formation, nitrogen fixing root nodules and autonomous crown-gall tumors on many higher plants including most dicots, some monocots and some gymnosperms (Matthysse, 2006). *Agrobacterium* is a Gram negative, aerobic soil borne bacteria has worldwide distribution (Furuya, *et al.,* 2004). *Agro bacterium* species are commonly known as bacteria that infect dicotyledonous plant from over 90 different plant families including economically important fruit and nut crops, grapes ornamental and landscape plants.

The occurrence of *Bacillus* species as endophytes has been reported from different plants such as pigeon pea (Selvakumar *et al.,* 2008), wheat, kudzu, and soybean nodules. They have been shown to benefit to their hosts by promoting nodulation and growth. Moreover, plant studies have shown that the beneficial effects of plant growth promoting microorganisms can be enhanced by co-inoculation with other microorganisms. Coinoculation frequently increased growth and yield compared to single inoculation. However, up to now, nodule endophytes of *S. alopecuroides* have not yet been specifically studied (Geetha *et al.,* 2008). Biosynthesis of Extracellular Polysaccharide (EPS) by *Rhizobium, Bacillus* and *Klebsiella* species is required for the formation of nitrogen fixing nodules on the indeterminate types of leguminous, such as *Leucaena, Medicago, Pisum, Vicia, and Trifolium* (Wilbert, *et al* 1998). Extracellular polysaccharides are the biological active compound and signaling molecule releasing from the nitrogen fixing bacteria from the root nodules. And the polysaccharides

produced by *Sinorhizobium meliloti* can mediate alfalfa root nodule invasion (Teplitski, *et al.,* 2002).EPS are extracellularly secreted microbial polysaccharides their amounts and chemical structure depend up on the microorganism and carbon substrate media. They can be classified on the basis of their composition in homo polysaccharides and hetro polysaccahrides. Homo polysaccharides consist of only one monosaccharide such as glucose, fructose, dextran. Hetro polysaccharides consist of two or more monosaccharides such as xanthan, galatose.

Extracellular polysaccharides or exopolymeric substances (EPS) are produced by bacteria and have an important function in the removal of heavy metals and radionuclides from wastewater and natural waters. For instance, EPS produced by specific bacteria have been used as biosorbents for toxic metals (Dhami *et al.,* 1998). Bacterial polysaccharides offer a viable alternative to the currently used traditional polysaccharides and find extensive application in food and pharmaceutical industries. Polysaccharides are incorporated into foods industry such as thickening, suspending or gelling agents in order to improve food quality and texture (Stephen, 1995).Polysaccharides from plant sources material and their modified forms such as starch, carageenans and Arabic gum as well as microbial polysaccharides are important additives in the food industry. These polysaccharides improve the textural properties and shelf life of bread and therefore are commonly used as additives for bread production. Polysaccharides have being used for a long time in the food industry as bio thickener, texture stabilizer or gelling agent. The industrial application of extracellular polysaccharides is the use of xanthan in oil industry (Linton, *et al.,* 1991). The most significant biomedical exploitation of bacterial polysaccharides is used as vaccine agent (Roberts, 1995).

In this regard, my present work was carried out in the plant *Tephrosia purpurea* selected for the production of Extracellular polysaccharides from isolated root nodule bacterium and HPLC analysis of extra cellular polysaccharides (EPS). This study concentrating on the anti bacterial activity of isolated nodule bacterium by Agar well diffusion method.

MATERIALS AND METHODS

Collection of plant sources

Tephrosia purpurea were collected along with the root nodule from the Udaya School of Engineering campus, Kanyakumari District, Tamil nadu, India.

Preparation of root nodule extract

The nodule were detached carefully and sterilized thoroughly by following standard procedure. The root nodules were kept immersed in 0.1 % acidified mercuric chloride solution for 5 minutes and washed repeatedly with sterile distilled water. Then they were immersed in 70% ethyl alcohol and washed with sterile distilled water. This treatment was followed by repeated washing with sterile distilled water. These sterilized root nodules were crushed simply with pestle and mortar and extracted with sterile distilled water.

Isolation of nodule endophytes

Bacterial species were isolated by serial dilution and pour plate techniques. The root nodule extract was serially diluted up to 10^{-6} to 10^{-9} with sterile distilled water. And 1ml of diluted sample was inoculated in to sterile Petri plates and poured with sterilized Yeast Extract Mannitol Agar (YEMA) medium. After inoculation the plates were incubated at 28^{o}C for 2 to 3 days. After incubation the bacterial colonies were purified by continuous streaking plate techniques on YEMA medium.

Antibacterial activity of isolated bacterium

Antibacterial activity of isolated bacterium was done to detect any antagonistic character against pathogens. The strains of *Escherichia coli, Enterococcus aeruginosa, Klebsiella*

pneumonia, and *staphylococcus aureus* were procured from CMFRI, Trivandrum, used for the antibacterial activity. Nutrient broth was used for cultivate the bacteria. Antibacterial activity was evaluated by using agar well diffusion techniques in petri dishes. Micro organisms were spread on Muller Hinton Agar (MHA) plates with the help of cotton sticks. Wells are made out of well cutter and added 200µl of supernatant to each well. Four petri plates were prepared in similar way for the four bacteria namely of *Escherichia coli, Entrococcus aerogenosa, Klebsiella pneumonia, and staphylococcus aureus.* After incubation for overnight at 37^{o}C, a clear zone of inhibition around a well was the evidence of antibacterial activity. Diameters of the zone of inhibitions were measured in millimeter.

Mass cultivation of sequenced species

For mass cultivation, the isolated species were grown in 500 ml conical flask at the temperature 30±2^{o}C, on a rotary shaker for 30 hours. The medium selected for the mass cultivation and bacterial growth was Yeast Extract Mannitol (YEM). When an appropriate cell density was reached, the cells were harvested from the culture by centrifugation. The growth pattern of isolated bacterium species was studied. The turbidity of growth was read at 540 nm colorimeterically.

Isolation of Extra cellular polysaccharides (EPS)

The bacterial culture was grown till it reaches stationary phase and harvested by centrifugation. After centrifugation at 1000 g for 20 minutes, the cell free culture was used for extracting extra cellular polysaccharides (EPS). Then three volumes of ethyl alcohol was added to the cell free culture fluid and again centrifuged at 6000 g for 10 minutes for precipitating polysaccharides. After centrifugation, the precipitated polysaccharides were collected and suspended in 1 ml distilled water. The dissolved polysaccharides were re precipitated with three volumes of ethyl alcohol. Finally, extra cellular polysaccharide in distilled water was used as sample.

Estimation of extra cellular polysaccharide (EPS) by UV-Vis spectrophotometer

The polysaccharide obtained in the above process was used for estimation of extra cellular polysaccharides by phenol sulphuric acid method. Reaction mixture in a test tube contained 1 ml of EPS solution and 1 ml of aqueous phenol and 5 ml of concentrated sulphuric acid was added. After vigorous shaking, the tubes were allowed to stand for 20 minutes. Then the absorbency was measured at 490 nm by using UV-Vis spectrophotometer. EPS solution in distilled water was used as control. The amount of extra cellular polysaccharides was determined against the starch standard before dialysis.

Extraction of Extracellular polysaccharides

After 30 hours of incubation, the cells were removed by centrifugation at 4000 g at 15 minutes, and 2 volume of chilled 95 % ethanol were added to the supernatant. After 3 hours of incubation at 4^{o}C, the precipitated polysaccharide was collected by centrifugation at 4000 g for 10 minutes. Then the pellet was re suspended in distilled water and again centrifuge at 8000 g for 10 minutes. Finally the supernatant was precipitated with ethanol.

Dialysis

The extracted polysaccharide was dialyzed using dialysis membrane-70 having 29.13 mm flat width 17.5 mm diameter. The required size of membrane was cut and washed with distilled water. Then the membrane was boiled for 5-10 minutes in 10mM sodium carbonate and 10mM EDTA solution and washed three times with distilled water. Then the membrane was boiled for 5-10 minutes in 10mM EDTA solution. Then the extra cellular polysaccharides were dialysed against deionized water at 4^{o}C for 24 hours.

Lyophilization
After dialysis process, the extra cellular polysaccharides produced were lyophilized and autoclaved and stored at room temperature.

High performance liquid chromatography (HPLC) analysis of EPS
The dialyzed samples were used for the HPLC analysis. The mobile phase used as water and flow rate was 0.7 ml/min. The column temperature was maintained at 80^{o}C. The dialyzed sample was filtered through a 0.2µm membrane before injection. Then 10µl of samples were directly injected and the retention time of the polysaccharides was determined.

RESULTS AND DISCUSIONS

Isolation of nodule endophytes
The plant *Tephrosia purpurea* was collected along with the root nodule from the Udaya School of Engineering campus, Kanyakumari District, Tamil Nadu, India. Five different colonies were isolated from root nodule extract. Then bacterial colonies were purified by continuous streaking plate techniques on YEMA medium.

Antibacterial activity of isolated species
Antibacterial activity of isolated bacterium was studied using the human pathogen *Escherichia coli, Enterococcus aeruginosa, Klebsiella pneumoniae*, and *Staphylococcus aureus.* Study was carried out by using agar well diffusion method showed in fig1.

The isolated species has high antibacterial activity against the gram negative pathogen *Klebsiella pneumonia* and *Escherichia coli* than gram positive pathogen *Enterococcus aeruginosa* and *staphylococcus aureus.* Isolated species D and E have maximum zone of inhibition against the pathogens. The zone of inhibition in mm showed in the table 1.

Identification of isolated bacterium
Two different colonies were selected and proved to be efficient against the various human pathogens. These two colonies were identified by 16s rRNA and the obtained sequences were found similar to different root nodule bacteria, namely *Bacillus licheniformis* and *Klebsiella pneumoniae.*

Growth study
The growth rate of isolated *Bacillus licheniformis* and *Klebsiella pneumoniae* was studied by measuring their absorbance in colorimeter at 540 nm for 30 hours. The exponential phase of *B.licheniformis* and *K.pneumoniae* was observed for first 18-20 hours. The growth curve of *B.licheniformis* and *K.pneumoniae* is given in graph 1 and 2.

Growth curve of *B.licheniformis* and *K.pneumoniae* was obtained. Both the graphs represent the four phases of growth in bacteria, namely, lag phase, log phase, stationary phase and decline phase. The stationary phase was observed in *B.licheniformis* at an interval of 18-22 hrs and in *K.pneumoniae* at an interval of 16-20 hrs respectively.

Isolation of Extra cellular polysaccharides (EPS)
Extra cellular Polysaccharides were isolated from *Bacillus licheniformis* and *Klebsiella pneumoniae*. Extra cellular polysaccharides isolated as a precipitate was obtained at the bottom of the centrifuge tube. Alcohol precipitation is the preferred method for isolating polysaccharide materials; the addition of the alcohol effectively lowers the solubility of the polymers, causing their precipitation (Smith *et al.,* 1982 and Vanhooren *et al.,* 1998). Alcohol precipitation was used to obtain the precipitate. By comparing with the method by Smith and Vahooren, the precipitate can be polysaccharide.

Estimation of extra cellular polysaccharides (EPS) by UV-Vis spectrophotometer
The isolated extra cellular polysaccharides were estimated by phenol sulphuric acid method. The amount of extra cellular polysaccharides from *Bacillus licheniformis* and *Klebsiella pneumoniae* were estimated by using UV-Vis

spectrophotometer before dialysis. Estimation of EPS from *B.licheniformis* and *K. pneumoniae* by UV-Vis spectrophotometer before dialysis is given in graph 3 and 4.

After the isolation of extra cellular polysaccharides, the high amounts of extra cellular polysaccharides are estimated 210 mg/ml from *K.pneumoniae* and 70 mg/ml from *B.licheniformis* before dialysis. Thus it was concluded the *B.licheniformis* and *K.pneumoniea* used for the extraction of extra cellular polysaccharides. The extracted extra cellular polysaccharides were purified by dialysis. After dialysis extra cellular polysaccharides were estimated from *B.licheniformis* and *K.pneumoniae* by UV-Vis spectrophotometer is given in graph 5 and 6.

After the dialysis the extra cellular polysaccharides were estimated, the high amount of extra cellular polysaccharides 12.3 mg/ml were estimated from *K. pneumoniae* and 10.97 mg/ml were estimated from *B.licheniformis*. *Bacillus species* and *Klebsiella species* produced various type of polysaccharides under static fermentation conditions. Most *bacillus* species are versatile chemohetrotrophs capable of respiration by using a variety of simple organic compound. Polysaccharides of *Klebsiella pneumoniae* and *Klebsiella oxytoca* have been intensity investigated for antigenicity. The extra cellulr polysaccharides produced by *K. pneumoniae* may participate in in vivo bacterial aggregation or adherence of host organisms. **Lyophilization**

After dialysis the extracted polysaccharides were produced by lyophilization. 50 ml dialyzed samples were lyophilized and 0.044 gm of EPS obtained from *B.licheniformis* and 0.061 gm of EPS obtained from *K. pneumoniae*. Thus it was concluded that *K.pneumoniae* was produced more amount of EPS than *B.licheniformis.*

High performance liquid chromatography (HPLC) analysis of EPS

The extracted polysaccharides sample was used for HPLC analysis. Three polysaccharides components were observed in HPLC chromatograms with their respective retention time. HPLC analysis of three polysaccharides is given in graph 7. And their retention time showed in table 2.

Bacillus species were easily isolated and readily grown in the bacteriology laboratory. The most accurate way to distinguish the member of genus *Bacillus* is by placing them into ecophysiological groups, such as nitrogen-fixers, denitrifying insect pathogens, animal pathogens, thermopiles and antibiotic producers. In addition, EPS usually consists of three to eight monosaccharides and the identified monosaccharides from the sample were two, lactose, isomaltoriose. EPS from lactic acid bacteria were galactose, lactose, glucose and rhamnose (Tevan ramanathan *et al*., 2011). By HPLC, polysaccharides of *B. licheniformis* were lactose and isomaltoriose. The identified monosaccharides from *K.pneumoniae* were glucose. By HPLC polysaccharides of *K. pneumoniae* was 56.04% galactose, 25.92% glucose, 10.96% galacturonic acid, 3.71% glucronic acid (Kuniho nakata *et al.,* 1999).

Figure1

Antibacterial activity of isolated species against the following pathogens 1. Escherichia coli, 2. Enterococcus aeruginosa, 3. Klebsiella pneumoniae and 4. Staphylococcus aureus

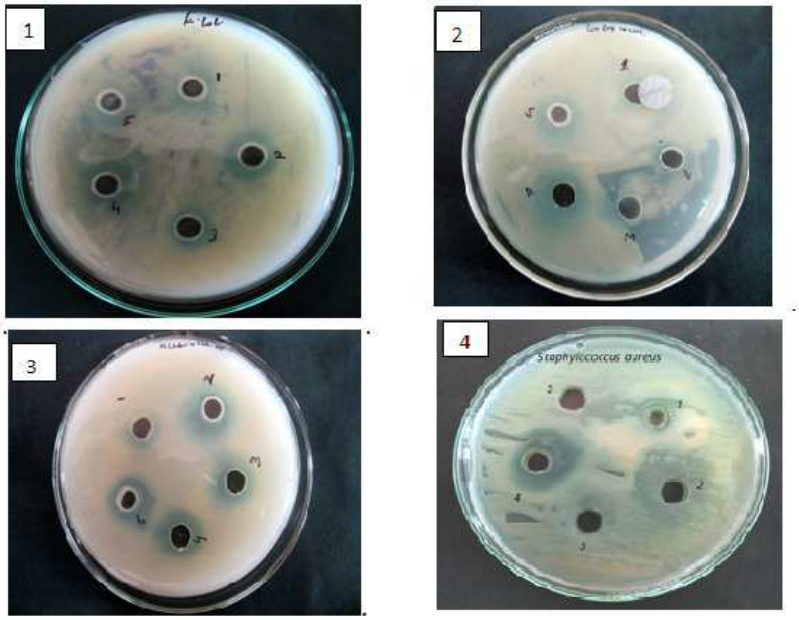


Table 1

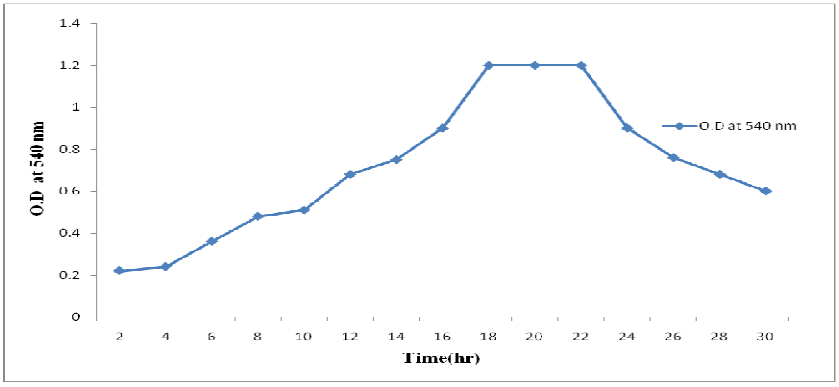
Antibacterial activity of isolated species against pathogenic organisms

Isolated species	Zone of Inhibition in mm			
	Enterococcus aeruginosa	*staphylococcus aureus*	*Klebsiella pneumoniea*	*Escherichia coli*
A	Nil	Nil	15	13
B	Nil	20	22	14
C	Nil	Nil	16	13
D	20	26	20	18
E	16	20	21	16

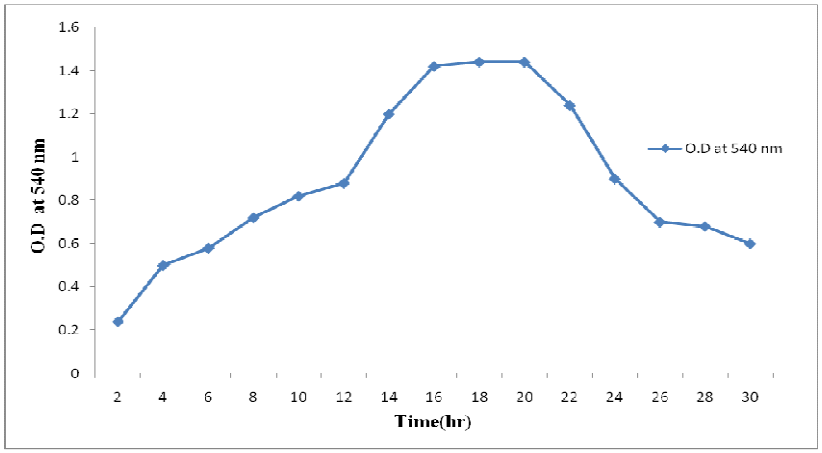
Table2

HPLC analysis of extra cellular polysaccharides (EPS) and retention time

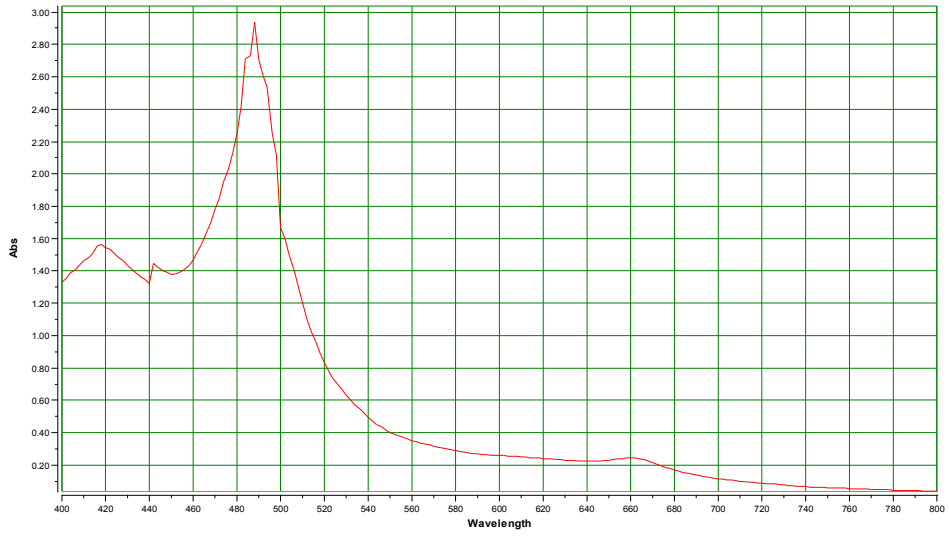
Sl. No	Components	Retention time(min)
1.	Lactose	3.15
2.	Isomaltoriose	8.55
3.	Glucose	11.27



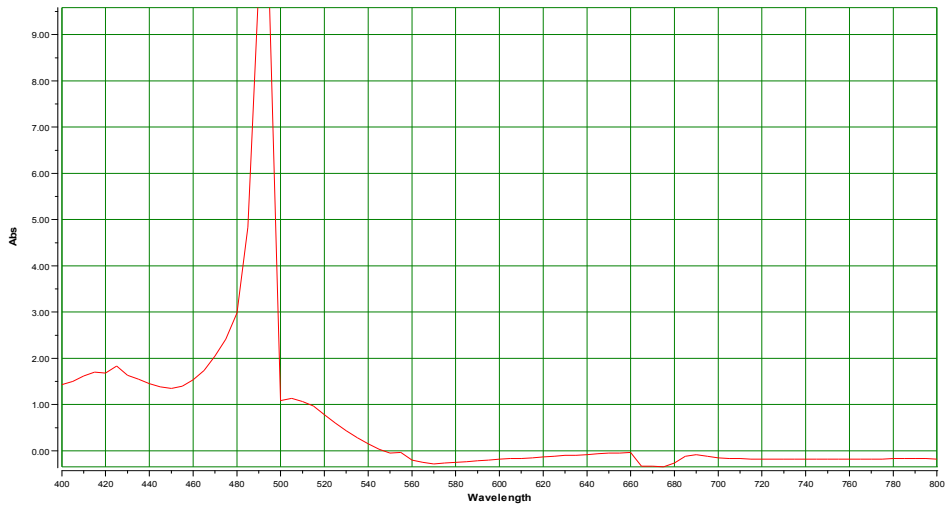
Graph 1
Growth curve of Bacillus licheniformis.



Graph 2
Growth curve of Klebsiella pneumoniae.

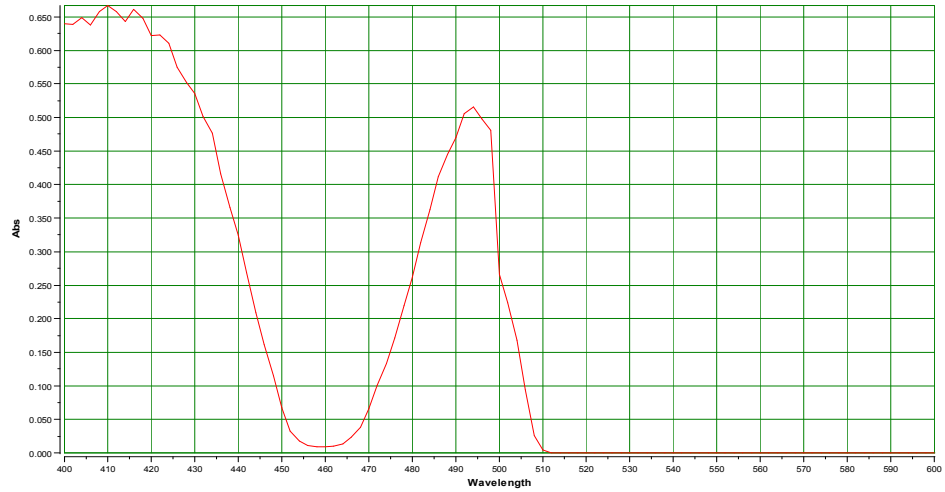


Graph 3
Estimation of EPS from Bacillus licheniformis by UV-Vis spectrophotometer before dialysis.



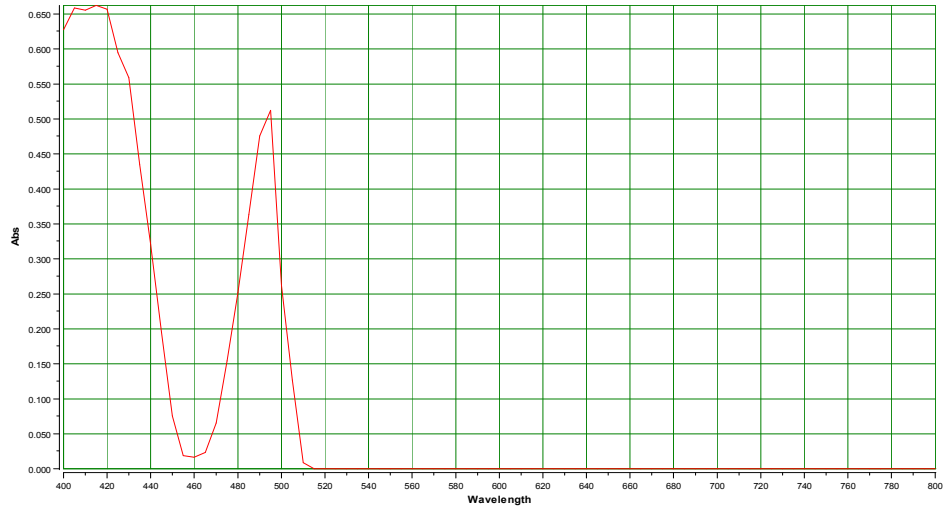
Graph 4

Estimation of EPS from Klebsiella pneumoniae by UV-Vis spectrophotometer before dialysis.



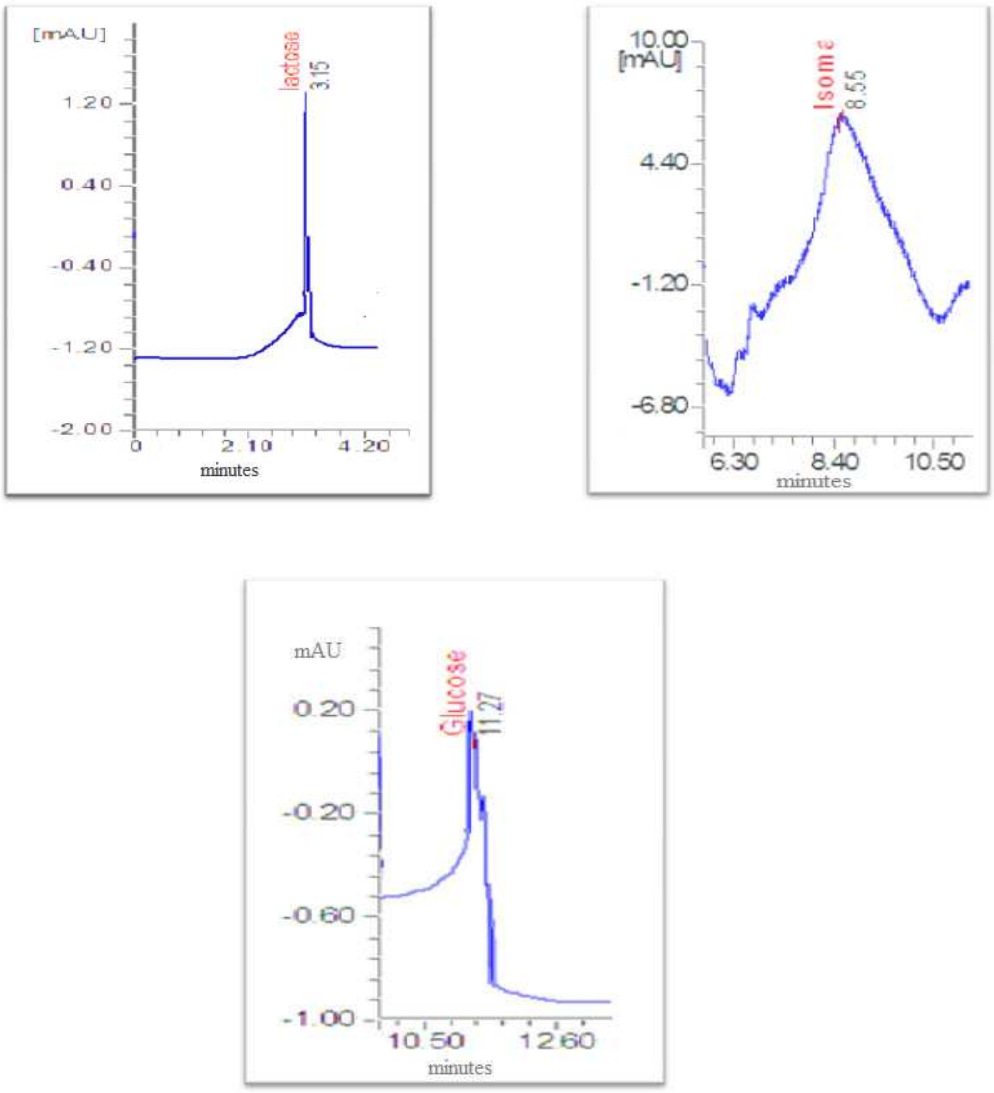
Graph5

Estimation of EPS from Bacillus licheniformis by UV-Vis spectrophotometer after dialysis.



Graph6

Estimation of EPS from Klebsiella pneumoniae by UV-Vis spectrophotometer after dialysis



Graph7

HPLC analysis of EPS from Bacillus licheniformis and Klebsiella pneumoniae

CONCLUSION

Five species of bacteria were isolated from the root nodules of *Tephrosia purpurea.* The isolated bacteria were purified in Yeast Extract Mannitol Agar (YEMA) medium by continuous streaking plate techniques. The *Bacillus licheniformis* and *Klebsiella pneumonia* has high antibacterial activity against *Escherichia coli, Enterococcus aeruginosa, Klebsiella pneumoniae*, and *staphylococcus aureus.* Mass cultivation of isolated *Bacillus licheniformis* and *Klebsiella pneumonia* was done by using Yeast Extract Mannitol (YEM) broth media. The growth curve and microscopic identification of *Bacillus licheniformis* and *Klebsiella pneumonia* were done. Extra Cellular Polysaccharides (EPS) were isolated and estimated from the each *Bacillus licheniformis* and *Klebsiella pneumonia.* Estimation of extra cellular polysaccharides was carried out by UV-Vis spectrometer analysis. The extra cellular

polysaccharides exhibited peak absorbance at 490nm. Before dialysis high amount of EPS of about 210 mg/ml was estimated from *Klebsiella pneumonia* and 70.02 mg/ml was estimated from *Bacillus licheniformi.* Then after dialysis high amount of EPS 12.3 mg/ml was estimated from *Klebsiella pneumonia* and 10.97 mg/ml from *Bacillus licheniformi.* By HPLC analysis three polysaccharides components lactose, Isomaltoriose and glucose were observed at their retention time 3.15, 8.55 and 11.27 minutes. Lyophilization was done for the production of extra cellular polysaccharides and 0.044 gm of EPS obtained from *B.licheniformis* and 0.061 gm of EPS obtained from *k.pneumoniae*

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