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Exopolysaccharide Production Optimization by Solid State Fermentation for Bacteria

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ABSTRACT
Bacteria have been used in numerous solid-state fermentations (SSF) from ancient times, nearly always in mixed culture. On the laboratory scale, a comparison between submerged culture (SC) and SSF for the generation of bacterial exopolysaccharide (EPS) revealed that the latter method produced 2 to 4.6 times more polymer than the former. Anti-microbial, anti-tumor, and anti-inflammatory properties have been described for microbial exopolysaccharides (EPSs). The antibacterial properties of EPS could be utilized to generate antimicrobial medicines, and they could have a variety of industrial, pharmacological, and medicinal uses. The present study was aimed to isolate efficient EPS producing bacteria. EPS producing strain grown in a Bushnell Haas medium (BHM) was isolated from soil sample and identified as Bacillus spp. Microorganism was separated by serial dilution method. Total 5 EPS producing isolates were obtained out of which, DS P5 found to be higher producers of EPS, hence DS P5 was given further considered. EPS production was observed higher at day 3 as compared to other days from DS P5 bacterial growth plate.
Keywords: Solid state fermentation (SSF), Exopolysaccharide (EPS), Bacterial isolation, Bushnell Haas Agar medium (BHM)

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INTRODUCTION

Solid state fermentation (SSF) or solid substrate fermentation is defined as a fermentation process that is occurring in the absence (or near absence) of free water [1]. However, the substrate must have sufficient moisture to support the growth and metabolism of micro-organism and it (SSF) promotes the growth of micro-organisms on moist substances in nature and is credited with the invention of fermentation technique in ancient time. As a result, it's not unexpected that practically all of the old fermentation procedures were based on SSF principles. SSF provides a variety of options for agro industrial residue processing. This is largely due to the fact that solid-state operations need less energy, produce less waste water, and are environmentally beneficial because they eliminate the problem of solid wastes [2]. On the laboratory scale, a comparison of SmF and SSF for the generation of bacterial exopolysaccharide revealed that the latter method produced 2 to 4.7 times more polymer than the former [3]. Bacterial EPS are extracellular compounds linked with adaptability, survival, and functions in living organisms (plants, animals, algae, bacteria, and fungi). It is used as thickeners, stabilizers, emulsifiers, binders, film formers, gelling agents, and suspending agents, that depending on their structural and rheological qualities behavior [4]. Over the last few decades, a large variety of bacterial EPS have been discovered, and their content, structure, production, and functions have all been studied and there has been a lot of research done on the properties. Despite the fact that a large number of molecular structures have already been described only a few bacterial EPS have been commercialized developed [5]. EPS, a high molecular weight value added substance, water soluble polymers, and linked together by glycosidic connections. It is secreted into the environment by various unique organisms. Because of its outstanding physicochemical qualities and unique chemical structure, EPS has been successfully used in a variety of industrial industries, including food, chemicals, petroleum, hydrocarbons, and vegetable oils, as well as mineral emulsification and environmental conservation [6]. Furthermore, they scatter in water to produce a

thickening or viscosity building effect due to their numerous fascinating physio-chemical and rheological features in that they disperse in water to give a thickening or viscosity building effect. Significant progress has been achieved in the search for novel microbial EPS with innovative and highly useful characteristics in recent years. The different biopolymers that have been considered and are currently being promoted as marketable products include hyaluronic acid from *Streptococcus equii* and *Streptococcus zooepidemicus ;* levan from *Bacillus subtilis* and *Bacillus polymyxa* ; pullulan from *Aureobasidium pullulans;* dextran from *Leuconostoc mesenteroides*; xanthan from *Xanthomonas campestris* ; gellan from *Sphingomonas paucimobilis ;* alginate from *Azotobacter chrococcum*; cellulose from *Acetobacter xylinium*; and succinoglycan from *Rhizobium* [7].

MATERIALS AND METHOD

Soil sample collection: - For bacterial isolation, 10 g of soil was collected from Roses Nursery, Vadodara district of Gujarat region in India, by using clean and dry sterile spatula in a clean polythene. Soil sample were collected from upper layer of the farmland where maximum population of microorganism was concentrated [8].

Isolation and purification of exopolysaccharide producing bacteria: - For reducing microbial population, 1g of soil was dissolved in 10 ml of sterile distilled water to make soil suspension. Isolates were obtained by serial dilution plating on Bushnell Haas Agar Medium (BHM) along with glucose by spread plate technique. 1 ml of each dilution (10^{-1} to 10^{-5}) of soil suspension was poured on BHM plates and spreaded over it by using sterile L rod. After incubation for three days hrs at 30°C, mucous colonies were observed on the plates. Isolates were maintained on BHM plates. Mucoid colonies were screened and re-streaked on another BHM plate to obtain pure culture. The Bacterial isolates were maintained on BHM agar slant and stored in refrigerator [9].

Exopolysaccharide production by solid state fermentation: - Isolated organisms were used for production of exopolysaccharides. 23.27 g of BHM was dissolved in 1000 ml distilled water and sterilized in autoclave for 15 min at 121°C. The medium consisting of the following components (g/l): Magnesium sulphate 0.200, Calcium chloride anhydrous 0.020, Potassium dihydrogen phosphate 1.000, Dipotassium hydrogen phosphate 1.000, Ammonium nitrate 1.000, Ferric chloride 0.050, Agar 20.000, and a final pH at 25°C is 7.0±0.2 [10]. To carry out solid state fermentation, streaking is done with the help of an inoculum loop on a fresh BHM media containing plate. Then it is transferred to an autoclave for three days at 30°C for further growth of mucoid colonies. After that the grown colonies then scrapped from the media plate with the help of a spatula. After collecting the colonies, it is then kept in a centrifuge tube along with 20 ml distilled water. The mixture is then centrifuged at 10,000 rpm (Rotation per minute) for 10 minutes to get supernatant. Then pour the supernatant into al blank beaker. After that 40 ml acetone is added to the supernatant for precipitation. The precipitation then poured into a blank plate to extract dry EPS. Extracted EPS was dried in hot air oven at 50°C and the correct weight of the precipitation determined by weighting the petri plate along and along with the dry EPS. The process is repeated for five different days to observe the trend [11].

Isolation and extraction of exopolysaccharide: - The cells were extracted by centrifugation at 10,000 rpm for 10 minutes. After centrifugation, 40 mL acetone was added along with the supernatant into a blank beaker, which was then kept at refrigerator at 40°C overnight. The precipitated material was collected and dried it at a temperature of 1000°C. After drying, the correct weight of the precipitation determined by weighting the petri plate along and along with the dry EPS to determine how much amount of exopolysaccharides produced from this plate. The process is repeated for five days to observe the trend [9].

Gram staining: - Gram staining is used for the characterization and identification of the organisms, that grown in BHM media plate. The Gram stain is by far the most popular method for dividing bacteria into two primary groups: Gram (+) positive and Gram (-) negative. Allow to air dry a thin film of specimen on a clean, grease-free slide. Pass it three times over a Bunsen flame to fix it. Allow 60 seconds for the crystal violet to soak into the film. To the slide, wash it off and apply iodine (mordant) to the stain for 60 seconds. Wash the slide and decolorize it with acetone (decolorizer) for a second, then wash it and train it with safranin (anti stain) for 60 seconds before washing it off. Then dry the back of the slide and air dry. After that, slide is examined with the oil immersion, 100X lens. A purple color signifies Gram (+) positive while the color of the safranin which is red signifies Gram (-) Negative [12].

RESULT AND DISCUSSION

For this study, soil samples were collected from Roses Nursery in Vadodara, Gujarat. For screening of effective EPS generating bacteria, the samples were serially diluted and plated. Here I took five media containing plates naming DS P1, DS P2, DS P3, DS P4, DS P5. The BHM plates had many bacterial colonies

after 72 hours of incubation at 30°C. Mucoid colonies were chosen and screened before being transferred to a fresh BHM media containing plate to obtain pure culture. Exopolysaccharide producing isolates were identified from a total of five mucoid colonies isolated from the soil sample (Figure 1). The soil sample isolates named DS P5 was discovered to be more potent EPS producers as compared to other soil sample isolates, hence DS P5 was given further considered for carrying out this study.

Effect of Days on EPS production: - In this study, it is observed that 5th plate naming DS P5 produce more mucoid colonies compare to other EPS producing plate. So, here I took DS P5 plate to continue further procedure. Since, the change in days affects the production of EPS, the bacterial isolate plate (DS P5) was streaked again on another five fresh BHM media containing plate to observed how much EPS produce on different days (that is day 1,2,3,4, and 5). And kept these all five plates at incubator for different days. Among them first plate is observed after 24 hours, second is observed after 48 hours, third is observed after 72 hours, forth is observed 96 hours and fifth plate is observed after 120 hours. The organisms were able to grow at different day but the bacterial growth and EPS production was observed higher at day three as compared to other days (Figure 2 & 3). As it observed that EPS growth started reducing after day three hence it is continued till day five and stopped due to decline in growth.

Extraction of EPS from EPS producing bacteria: - After 24 hours the grown colonies from the first media containing plate are collected with the help of a spatula. After 48 hours this process is repeated for second plates. Then after 72 hours process is repeated for third plate. And so on till the fifth plate. After collecting the colonies, it is then kept in a centrifuge tube along with 20 ml distilled water. The mixture is then centrifuged at 10,000 rpm (rotation per minute) for 10 minutes to get supernatant. This process is repeated for fest of the plates. Then pour the supernatant into at different five blank beaker from five centrifuge tube. After that 40-60 ml acetone is added to each and every beaker to the supernatant for precipitation. The precipitation then poured into different blank plate from different beaker to extract dry EPS. At the mean time need to weight each and every blank plate and along with the precipitation for knowing the exact weight of EPS.

Extraction of dry EPS: - Extracted EPS of different plate was dried in hot air oven at 50°C and the correct weight of the precipitation determined by weighting the petri plate along and along with the dry EPS. The process is repeated for rest of the plates, to observe the trend of EPS production from different five days. The EPS from all five days were successfully extracted in the dried form and weighed (Table 1). Among them in day three was found to be higher producers of EPS with 0.29g production from 20 ml distilled water (Figure 4). Figure 5 that shows the graphical representation of EPS production in different days.

Gram nature: - For performing gram staining sample are taken from DS P5 plate and followed the process of gram staining. The slide was examined under electron microscope using 100X magnification. Medium size short roads are observed along with pink colour. Hence it is a gram-negative bacterium that is *Bacillus* (figure 6).

In this research it is observed that here exopolysaccharide production is good from soil sample of nursery. Exopolysaccharide is produced from *Bacillus*. Here EPS is produced more in day three that is 0.29 g from 20 ml distil water. But in another paper their EPS is produced from *E. Coli* and production rate is less than *Bacillus* that I observed. EPS production is varied on pH. The change of pH effects the production of EPS. The bacterial isolates were streaked on media containing at same pH range that is 6.0. In another paper the bacterial isolates were streaked on media plate at different pH (6.0, 7.0, 8.0 and 9.0). And there it is also found that EPS production is high at pH 6.0. So, it is said that production of EPS depends upon days and pH.

TABLE 1: Below is the stage wise observation of EPS production from DSP5 plate in different days,

Sample ID	Duration	Produced Qty	Unit Of Measure
DSP5	Day 1 (24Hrs.)	0.02	g/20ml
DSP5	Day 2 (48 Hrs.)	0.03	g/20ml
DSP5	Day 3 (72 Hrs.)	0.29	g/20ml
DSP5	Day 4 (96 Hrs.)	0.18	g/20ml
DSP5	Day 5 (120Hrs.)	0.12	g/20ml

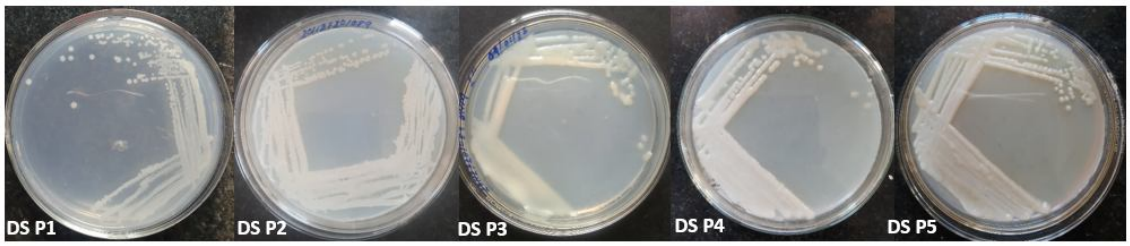

FIGURE 1: Isolation of EPS producing bacteria: Bacterial Colonies were isolated from dilution soil samples. Numerous colonies were obtained out of which total five mucoid isolated colonies were selected from higher dilution plates and designated as exopolysaccharide producing isolates.

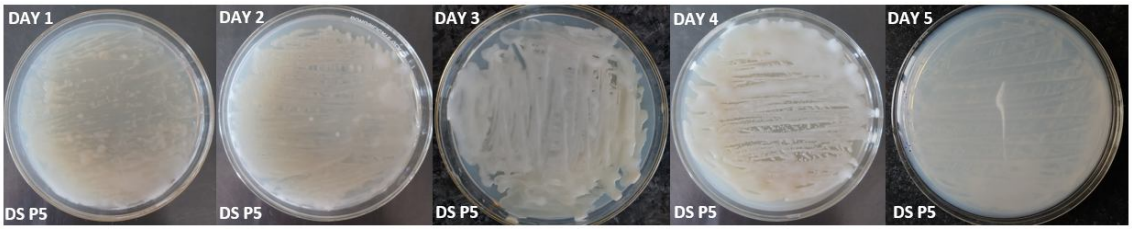

FIGURE 2: EPS producing bacterial plate (DS P5) isolates at different day (1,2,3,4 and 5): The bacterial isolates growth was found to be optimum at day 3 along with the good EPS production.

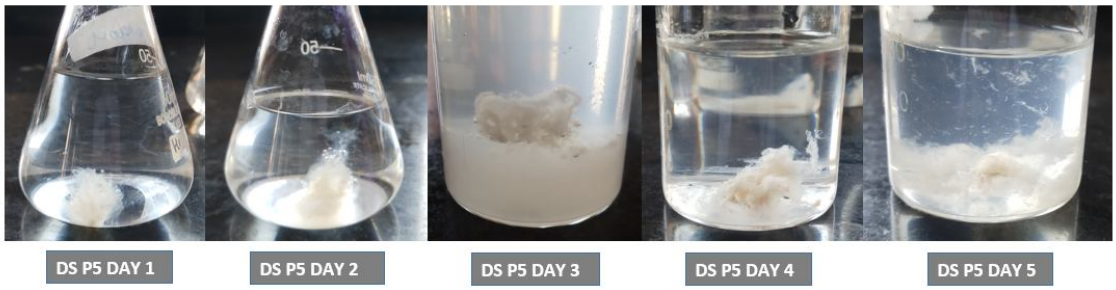

FIGURE 3: EPS producing bacterial plate (DS P5) produced a good amount of EPS at different days (1,2,3,4 and 5). Optimum growth is found at day 3

FIGURE 4: Dried form of EPS production in different days

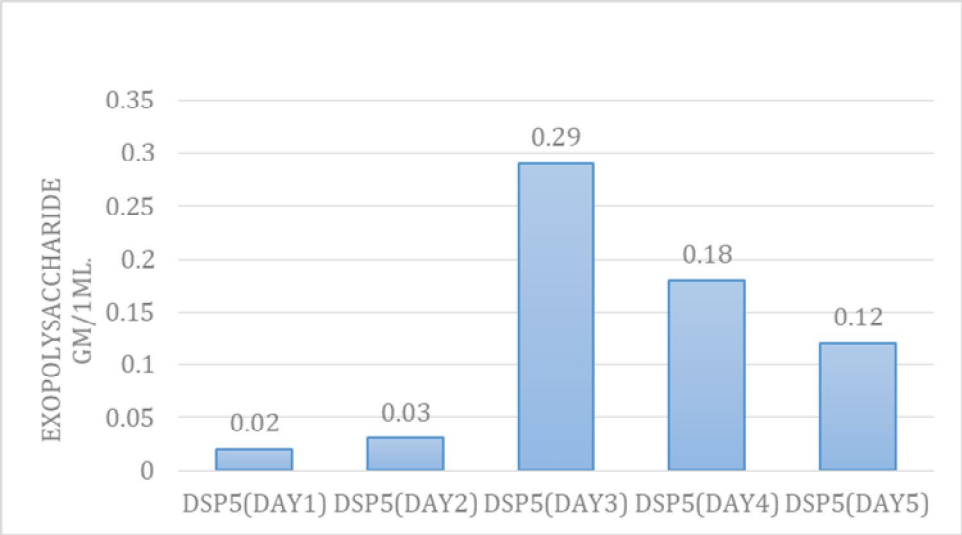

FIGURE 5: Graphical representation of exopolysaccharide production in different days

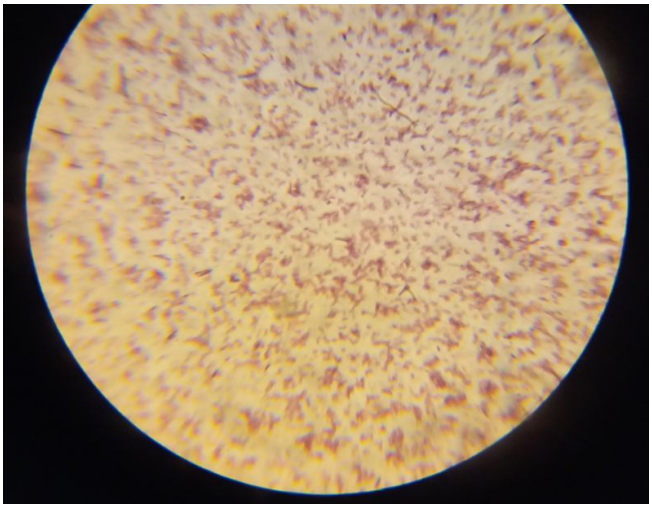

FIGURE 6: 100X microscopic view that is *Bacillus* spp

CONCLUSION

The results of the present study recommend that bacteria isolated from soil sample are found to be good EPS producers. In particular, DS P5 was found as efficient mucoid colonies producers with the higher of EPS as compared to other plates. So, I took DS P5 for continue further study. DS P5 was streaked to another five media containing plate to observe the trend the EPS production. It is observed from five days, among then in day three produced a good amount EPS from DS P5 compare to other four days. As it observed that EPS growth started reducing after day three hence it is continued till day five and stopped due to decline in growth. However, further research that is TLC (Thin layer chromatography), FTIR (Fourier transform spectroscopy), HPLC (High performance liquid chromatography) is necessary to know the character of above-mentioned plate of EPS.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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