Research Article

Production and Optimization of Exopolysaccharides (EPS) Using Low-cost Bagasse as Substrate by *Lysinibacillus macroides*

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ABSTRACT

Exopolysaccharides are the secondary metabolites produced by many microorganisms and play a role as biopolymers extensively to protect living cells in various ways. In the present study, the carbon source was substituted by various cheaper agricultural wastes, investigated for EPS production, and found bagasse more suitable substrate than others. Bagasse at a concentration of 5% gave the highest EPS production of 13.13 ± 0.44 g L^{-1}. The effect of various parameters like incubation period, agitation speed, inoculum size, pH, and the temperature was also studied and found to have the highest EPS production after an incubation time of 120 hr (15.10 g L^{-1}), agitation speed of 100 rpm (12.47 g L^{-1}), 10% inoculum size (13.43 g L^{-1}), pH 7 (11.47 g L^{-1}) and incubation temperature 30°C (13.17 g L^{-1}). Thus, the present study confirmed that agricultural wastes could be a cheaper substitute for synthetic and expensive carbon substrates, which economically opens a new door toward EPS production.

Keywords: Agricultural wastes, Bagasse, Exopolysaccharides, Optimization.

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INTRODUCTION

Exopolysaccharides (EPS) are organic macromolecules produced by micro-organisms, plants and animals by utilizing any carbon sources. They are polymers of different monomers like monosaccharides, amino sugars, and uronic acids, as well as some non-carbohydrate substituents like amino acids, nucleic acids, phospholipids, etc. and are functioning as an energy reserve material. They give mechanical shape and rigidity to living cells.[1] They protect the cells against desiccation, osmotic stress, predation, toxic compounds, antibiotics, etc.[2] They can perform various diverse functions also, such as emulsification and degradation of hydrocarbon pollutants,[3-6] metal bioleaching,[7] antimicrobial shield production,[8] cryoprotectants,[9] and biofouling.[10]

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Generally, EPS-producing organisms are isolated from environmental conditions having a higher ratio of carbon-to-nitrogen in complex media or chemically defined synthetic media. These organisms are identified as EPS producers on the basis of mucoid colony characteristics.[11] These complex media are very expensive and affect EPS production economically. So, to reduce EPS production costs, many investigators have carried out research works to find out cheaper agricultural wastes for the production of EPS. The research studies of many agricultural wastes like bagasse,[12-16] cane molasses,[17-19] rice bran,[17,20,21] coconut waste,[22] groundnut shell,[23,24] mango peels,[25] fruit and potato wastes,[1] corn wastes,[26,22] etc. as a substitute for carbon sources have been reported for EPS production. Sugarcane is grown in large amounts all over India. Bagasse, an agro-waste, is the outcome after the processing of sugarcane and is disposed of indiscriminately to create a big nuisance to nature. But it can be a useful cheaper substance as a substitute for

carbon source for EPS production in synthetic media. In this study, bagasse was used as the sole source of carbon for EPS production by *Lysinibacillus macroides*. Some other agricultural wastes were also taken for comparative study. Optimization of various important factors, which can possibly affect EPS production, such as bagasse concentration, incubation period, agitation speed, inoculum size, pH and temperature, were also investigated.

MATERIALS AND METHODS

Micro-organisms and culture conditions

Lysinibacillus macroides, the organism used in this study, was isolated from rhizosphere soil by ancient microbiological techniques. The organism was grown in Modified Yeast Extract (MYE) medium containing (g L^{-1}) sucrose, 30; yeast extract, 1; KH_2PO_4, 1; $MgSO_4$, 0.5; pH, seven at 30±2°C for 96 hr.[1]

Pretreatment of agriculture residues

Different agriculture residues like bagasse, coconut waste, groundnut shell, rice bran and wheat husk were taken for this study. Bagasse and coconut waste were dried at 100°C to remove moisture, crushed, sieved through 1 mm, autoclaved (10% w/v) and filtered through Whatman No.1 filter paper. Groundnut shell, rice bran and wheat bran were dried, crushed and sieved similarly. Prepared 10% w/v solution and added 0.5% each of amylase, proteinase, pectinase, lipase and laccase enzymes. The mixture was heated at 80°C for 30 min and filtered through Whatman No.1 filter paper. These filtrates were used as a replacement for sucrose in MYE medium for EPS production.

Evaluation of different agriculture residue as a substrate

50 mL of MYE medium containing 3% each of agriculture residue was inoculated with 10% v/v inoculum and incubated at 30±2°C for 96 hr on a rotary shaker (120 rpm).[27] Then proteins were precipitated by adding Trichloroacetic Acid (TCA) (5% w/v) and agitating for 30 min at room temperature.[28-31] Protein precipitates were separated by centrifugation (10,000 rpm) for 20 min. The supernatant was collected; three volumes of ice-cold ethanol were added and kept overnight at 4°C to precipitate EPS in the medium. EPS was collected by centrifugation as above, given three washes with ice-cold ethanol, dried (65°C) and weighed.[1] One flask of MYE medium containing sucrose was taken as a control.

Optimization of growth conditions

The agriculture residue suitable for the highest EPS was taken to optimize different growth conditions. In this study, the production of EPS was optimized in different bagasse concentrations (1, 2, 3, 4, 5, 6 and 7%). The effect of incubation time (24, 48, 72, 96, 120, 144, 168 hr), agitation speed (0, 50, 100, 150 and 200 rpm) and inoculum size (1, 5, 10, 15 and 20%) on the production of EPS were also studied here. Various physical parameters like pH (6.5, 7, 7.5 and 8) and temperature (20, 30, 37 and 45°C) were optimized too for EPS production.

RESULTS

EPS production using different agriculture residues as a substrate

Many EPS-producing microorganisms can grow in relatively complex media containing appropriate nutrient sources like yeast extract, corn-steep liquor, starch hydrolysate, sucrose, etc. Some microbial species have the ability to utilize different agricultural wastes as a substitution for these nutrient sources for EPS production.[32] In the present study, various agriculture residues like bagasse, coconut waste, groundnut shell, rice bran and wheat husk (3% each) were replaced in the growth medium as a sole source of carbon. Pretreatment of these wastes was necessary because sometimes they contain very complex nature or inhibitory substances which can affect EPS production. Sometimes coloured compounds also need pretreatment. Results shown in Table 1 revealed that the production of EPS was significantly higher in bagasse (5.53 ± 0.28 g L^{-1}), the cheapest substitute as a carbon source, followed by wheat husk (3.33 ± 0.31 g L^{-1}).

Optimization of EPS production

EPS production at different bagasse concentrations

Production of EPS varies with the changes in carbon source concentration. So it was very necessary to find out the optimum concentration of carbon source in the growth media. Different concentrations of bagasse (1 to 7%) were taken to evaluate the optimum concentration. The highest EPS production (13.13 ± 0.44 g L^{-1}) was obtained with a 5% bagasse concentration (Table 1). It was observed that EPS production increased gradually up to 5% bagasse concentration and then decreased at a further increase in bagasse concentration.

Effect of incubation time

EPS production was measured up to 168 hr at an interval of 24 hr. Results revealed that the production

Table 1: EPS production in the growth medium containing different agriculture residues and at different bagasse concentrations.

Parameters	EPS production (g L^{-1})*
Agriculture residue (3%)	
Bagasse	5.53 ± 0.28
Coconut waste	1.93 ± 0.20
Groundnut shell	2.70 ± 0.17
Rice bran	2.07 ± 0.19
Wheat husk	3.33 ± 0.31
Bagasse concentration (%)	
1	3.77 ± 0.28
2	4.53 ± 0.20
3	5.87 ± 0.18
4	8.43 ± 0.33
5	13.13 ± 0.44
6	7.37 ± 0.48
7	4.23 ± 0.44

*Results are the average of trials done in triplicates ± standard deviation.

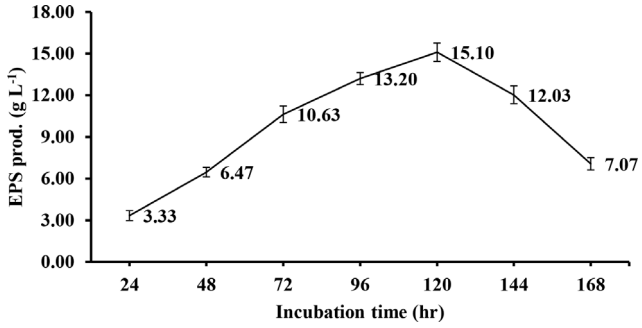

Figure 1: Effect of incubation time on EPS production.

of EPS increased up to 120 hr and then reduced (Figure 1). The optimum EPS was obtained at 120 hr (15.10 g L^{-1}) followed by 96 hr (13.20 g L^{-1}).

Effect of agitation speed

EPS was produced at a specific agitation speed. At higher agitation speed, the production of biomass increases and it affects the production of EPS. Same way, if the agitation speed was lowered, the synthesis of EPS was affected. Results indicate that 100 rpm was the optimum agitation speed for the EPS production (12.47 g L^{-1}) by *Lysinibacillus macroides* (Figure 2). EPS production was also studied in static conditions and found a small amount of EPS production (2.77 g L^{-1}).

Effect of inoculum size

Different inoculum sizes were studied for EPS production. On the basis of the results shown in Figure 3, it was found that the EPS production was maximum

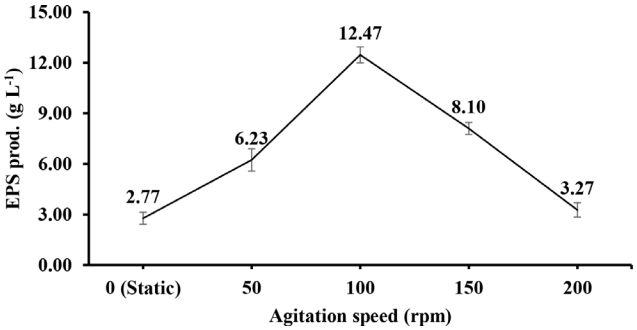

Figure 2: EPS production at a different agitation speed.

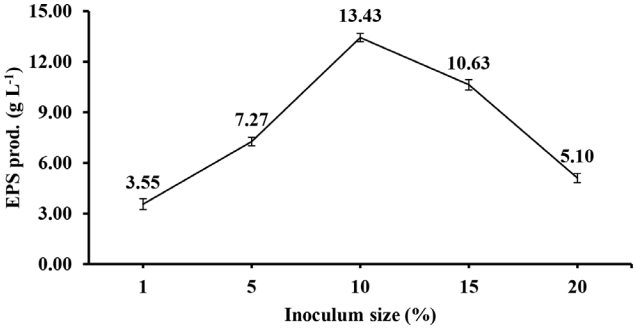

Figure 3: EPS production with various inoculum sizes.

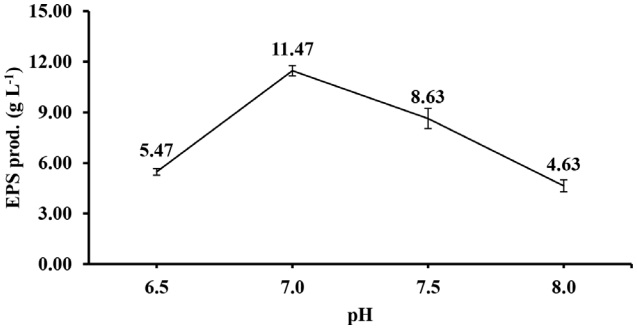

Figure 4: pH affecting the production of EPS.

at 10% inoculum size (13.43 g L^{-1}). If the inoculum size was increased, crystallization of the medium occurred, which might be due to the higher biomass concentration.

Effect of pH

pH is the crucial factor affecting EPS production. As the pH increased, the production of EPS increased progressively and reached a maximum of 11.47 g L^{-1} at optimum pH of 7. At a pH value of 8, a significant reduction in EPS production was detected, as shown in Figure 4.

Effect of incubation temperature

EPS production was studied by incubating growth medium at different temperatures. Figure 5 showed that the lower temperature (30°C) was suitable for growth

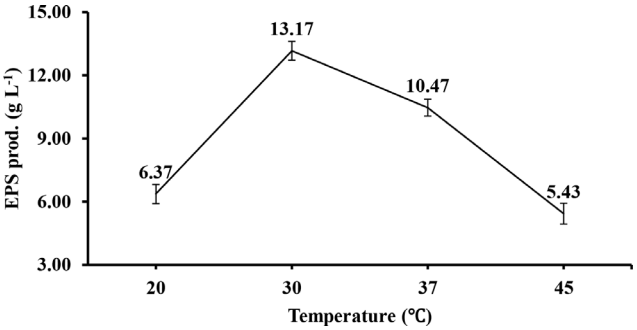

Figure 5: Production of EPS at different temperatures.

and EPS production (13.17 g L^{-1}) by *Lysinibacillus macroides* compared to the higher temperature.

DISCUSSION

Many critical factors affect EPS production, like carbon and nitrogen source, inoculum size, agitation speed, incubation time, pH, temperature, etc. The changes in these culture conditions directly affect the quantity and quality of the microbial EPS. The optimization study was useful to evaluate the effect of different parameters on the simultaneous increase in EPS production.[21] The results summarized that the bagasse could be a cheaper substitute for EPS production with a 5% concentration. The cultural conditions were also optimized successfully for the highest EPS production using bagasse as a substrate.

Our results of bagasse as a substitute are similar to the results reported by Getachew and Woldesenbet,[33], who obtained 5.00 ± 0.08 g L^{-1} EPS from *Bacillus* spp. Some researchers also found more EPS production than us (18 to 20 g L^{-1}) using bagasse as a substrate in the past.[13,14] Pawar *et al.*[27] reported 5.20 g L^{-1} EPS production after 72 hr of incubation, similar to the results obtained by us, but some researchers also reported higher incubation times of up to 9 days for fungi.[34]

Our optimization result for agitation speed is correlated with the results reported by Yang and Liau[35] for maximum EPS production on 4th day of incubation. In contrast, many researchers also obtained higher EPS production at 200 rpm speed.[36,37] Vaishnav, A.M.[1] also reported maximum EPS at 250 rpm agitation speed. Our results of 10% inoculum size were confirmed by the results obtained by Vaishnav, A.M.[1] and Maalej *et al.*[38] Some past investigations also confirmed that the range of inoculum size varies from 3 to 10% depending on the culture used for the study.[25,39,40]

We have obtained an optimum pH condition of 7 for maximum EPS production (11.47 g L^{-1}). Similar results were reported by Getachew and Woldesenbet[33] (12.00 g L^{-1}) at pH 7. Some researchers found higher EPS production also up to 33.00 g L^{-1} at pH 7 in the past.[41] In optimization of temperature, maximum EPS production was obtained at 30°C (13.17 g L^{-1}), which was similar optimum temperature reported by Hector *et al.*[16] for maximum EPS production. The probability of this higher EPS production might be due to the reduction in enzymatic activity responsible for EPS synthesis and/or growth rate.[21] Lower temperature makes more precursors available for EPS synthesis, along with a decrease in growth rate and cell wall polymer synthesis.[20] Khani *et al.*[42] also reported 14.50 g L^{-1} EPS production at 33°C.

The results and discussion of this one-factor-at-a-time study primarily confirm that bagasse can be an alternative to synthetic and complex carbon sources. Further optimization study by response surface methodology has to be carried out to obtain more optimized culture conditions for EPS production. It can reduce the production cost of the EPS in large-scale production.

CONCLUSION

Among the five agricultural wastes used, bagasse showed a significant amount of EPS production of 5.53 ± 0.28 g L^{-1} followed by wheat husk (3.33 ± 0.31 g L^{-1}). The least EPS production was obtained in the medium containing coconut waste (1.93 ± 0.20 g L-1). In the optimization study, the highest EPS production was obtained in media of pH 7 after 120 hr with an inoculum size of 10%, incubated at 30°C with agitation at 100 rpm. Results clearly indicate that bagasse could be used as a cheaper source for EPS production.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

EPS: Exopolysaccharides; **MYE:** Modified Yeast Extract; **KH_2PO_4:** Dihydrogen Potassium Phosphate; **$MgSO_4$:** Magnesium Sulphate; **TCA:** Tri-Chloro Acetic acid.

SUMMARY

The present study summarises that agricultural waste like bagasse can be a substitute for EPS production at 5% concentration, and maximum EPS production was obtained after 120 hr of incubation time with 10% of inoculum size at pH 7 at 30°C and 100 rpm. So further studies can be done with bagasse to make it a commercially cheaper source for EPS production.

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