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(RESEARCH ARTICLE)

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Analysis of bacterial growth parttern in bioprocessing of polyhydroxyalkanoates from waste oil by *Pseudomonas oleovorans*

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Abstract

Pseudomonas oleovorans NCIMB 6576 and *Ralstonia eutropha* NCIMB 10442 were used for the production of Polyhydroxyalkanoates (PHA) from industrial waste cooking oils, the bacteria were cultured on tryptone soya broth (TSB) and Tryptone soya agar (TSA). The growth pattern of the bacteria, serial dilution and viable counting was done using the Miles and Misra method, 0.5ml (500 µl) of the sample was transferred aseptically into test tubes filled with 4.5ml ringer solution (1/4 strength) resulting in a ten-fold dilution, the growth curve of the cultures of *P. oleovorans* NCIMB6576 grown on TSB with and without PS oil sample shows error bars in the graph for each point depicting the standard error of the mean. The initial viable count ranges between 6.37 log10 cfu/ml and 5.1 log10 cfu/ml. The viable count reached its peak after 30 hours giving approximately 9.7 log10 cfu/ml for *P. oleovorans* NCIMB6576 with PS oil and 9.24 log10 cfu/ml after 30 hours as well without the oil, showing that maximum cell count was attained at the same time. The growth curves of *P. oleovorans* NCIMB6576 grown on TSB with and without the oil sample TS, where the errors bars depicts the standard errors of the means on each point. The initial viable count at the start of the experiments shows that for *P. oleovorans* NCIMB6576 grown with the oil, there was an initial viable count of 6.1 log10 cfu/ml as compared to 5.1 log10 cfu/ml without the oil respectively. It was observe that the time at which maximum cell counts was attained is slightly longer when the oil was not used as a carbon source (30 hours) as compared to the oil control (27 hours). A decline in cell count is also noticeable after 30 hours until it reaches its minimum value of 9.4 log.10 cfu/ml after 48 hours in the experiment involving the oil sample TS.

Keywords: Polyhydroxyalkanoates; Waste Oil; *Pseudomonas oleovorans*; Bacteria; Growth

1. Introduction

Synthetic polymers derived from petrochemicals are not biodegradable and their indiscriminate disposal results in increased environmental pollution problems notwithstanding their diverse domestic. The resistance of these synthetic plastics to degradation coupled with their disposal has attracted attention to develop alternative biodegradable polymers that are compatible with the while retaining the physico-chemical properties of the conventional plastics. Such physical properties may include crystallinity, melting point, density, tensile strength and glass transition temperature which vary when compared to conventional plastics (Akaraonye *et al*., 2010) and can be modified through blending with either the natural or synthetic polymers. Polyhydroxyalkanoates (PHAs) are group of biodegradable polymers of hydroxyalkanoates (HAs) produced by various microorganisms through the accumulation of energy storage materials to overcome stress (Sexana and Tiwari, 2011). This view was supported by Kesharvarz and Roy (2010), indicating that various gram positive and gram negative bacteria are capable of producing the polymer in limited supply of nitrogen, phosphorus or oxygen in the presence of excess carbon sources. The PHAs are synthesized through polymerization of hydroxyacyl-CoA thioesters in a reaction catalysed by PHA synthases.

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The use of waste materials obtained from agriculture and industries as starting materials for the biosynthesis of PHA is an important strategy for the cost effective production of the polymer and provides an alternative means to overcome waste disposal problems. Different convertible substrates obtainable from variety of sources that includes waste cooking oil and plants oils provides triacylglycerols or fatty acids and glycerol which can serve as direct carbon source after hydrolysis of the constituent triacylglycerol backbone (Koller *et al*., 2010). Food industry wastes including oils mainly disposed as wastes in the environment can serve as carbon sources for the microbial synthesis of PHAs, an important bioplastic material (Obruca *et al.,* 2010). In this research, *Pseudomonas oleovorans* NCIMB 6576 and *Ralstonia eutropha* NCIMB 10442 were used for the production of PHB from industrial waste cooking oils.

2. Material and methods

2.1. Microorganisms

Pseudomonas oleovorans NCIMB 6576 and *Ralstonia eutropha* NCIMB 10442 were used for the production of PHB from industrial waste cooking oils. These microorganisms were collected from the culture collection of microbiology laboratory, University of Wolverhampton, UK as streaked plates. The microorganisms were refrigerated at 4^{o}C and cultures were maintained by streaking on TSA plates, once after every two weeks.

2.2. Industrial waste oil samples

Two industrial waste oil samples (supplied by Living fuels company, Kings Street, Nottingham) coded PS and TS were utilised individually as carbon sources for PHA production.

The oil samples differ in their fatty acids (FA) compositions with varying range of monounsaturated, polyunsaturated and saturated fatty acids as highlighted by the iodine number of each sample as shown in table 4a and b

Table 1a The fatty acid composition of the oil samples and 1b, showing the iodine number of the oils used as carbon sources in this research

A	Fatty acids	TS oil (g/100g)	PS oil (g/100g)
	Saturated	11.06	9.72
	Monounsaturated	48.76	59.30
	Polyunsaturated	35.78	26.58

B	Oil sample	Iodine number
	TS	106.2
	PS	98.1

2.3. Growth media

The growth media used in this experiment are tryptone soya broth (TSB) and Tryptone soya agar (TSA). Tryptone soya broth (Lab M, UK) consist of water, 17g/l trptone, 3g/l soy peptone, 5g/l NaCl, 2.5g/l K_2HPO_4 and 2.5 g/l D-glucose. This was prepared by dissolving 30g/l of the powder in 1L of distilled water and stirring with a magnetic stirrer (VELP scientifica). 250ml of the broth was then transferred into 500ml conical flasks and autoclaved at 121^{o}C before use.

TSA (Lab M, UK) contains distilled water, 15g/l tryptone, 5g/l soy peptone, 5g/l NaCl and 12g/l Agar No.2. The agar was prepared by dissolving 37g/l TSA powder in 1L of distilled water (equivalent to 7.4g in 200ml of distilled water in separate bottles). These were autoclaved at 121^{0}C and poured aseptically into Petri dishes and allowed to cool so that it becomes solid.

Ringer solution (Lab M, UK) was used for serial dilution (1/4 ringer) by dissolving one tablet in 500ml distilled water and dispensed into test tubes before autoclaving at 121^{o}C. The solution aids in keeping the cells alive before they are plated on agar.

2.4. Starter preparation

To prepare the starter culture which was used for the fermentation experiment, a single colony of *P.oleovorans* NCIMB 6576 or *R.eutropha* 10442 was inoculated into a conical flask filled with 20ml TSB. This was incubated in a rotatory shaker (Brunswick Scientific, UK) at 30°C and 150rpm for 24 hours.

2.5. Sonication

To create a homogenous mixture of the oil and TSB, the mixture was sonicated to break the oil into fine droplets to enhance its miscibility. 50ml of TSB was transferred into a sterile beaker to which 2.5ml of oil was added (equivalent to 2.5g) and sonicated for 10 minutes at 0.5 active and 0.5 passive intervals with 50% power using a sonicator (Bandelin sonopulz). This was then transferred aseptically back into the 500ml conical flasks to form a broth-oil mixture (with initial oil concentration of 10g/l). This procedure was carried out for each flask since all experiments were carried out in triplicates.

2.6. The fermentation process

Batch fermentation in conical flasks was carried out by preparing 250ml of TSB into each flask (of which 50ml of it was initially sonicated with 2.5ml oil to form a homogenate). The initial oil concentration in the mixture was approximately 10g/l. The oil- broth mixtures in the flasks were inoculated with 100µl of the starter culture. The shake flasks were then incubated in a rotatory incubator at 150rpm, 30°C for 48 hours. However, the fermentation was stopped at intervals to enable sample collection at time (0, 3 and 6, 24, 27, 30 and 48 hours).

Figure 1 Flask containing the fermentation culture (mixture of oil, broth and bacteria)

2.7. Serial dilution and viable count

To monitor the growth pattern of the bacteria, serial dilution and viable counting using the Miles and Misra method was employed. 0.5ml (500 µl) of the sample was transferred aseptically into test tubes filled with 4.5ml ringer solution (1/4 strength) resulting in a ten-fold dilution. This was further serially diluted to eight times (-1 to -8) in eight separate test tubes. TSA plates were divided into sectors -1 to -8 using two plates of four sectors each. 20 µl of each dilution in the test tubes was then transferred aseptically onto corresponding sectors labelled on the TSA plates using a Finn pipette after mixing with a vortex mixer. This procedure was carried out in triplicate for each dilution so that triplicate count can be conducted. Drops dispensed onto the TSA plates were allowed to dry before the plates were incubated for 24 hours at 30°C in an incubator. The colony growth on TSA plates is shown in figure 8 below.

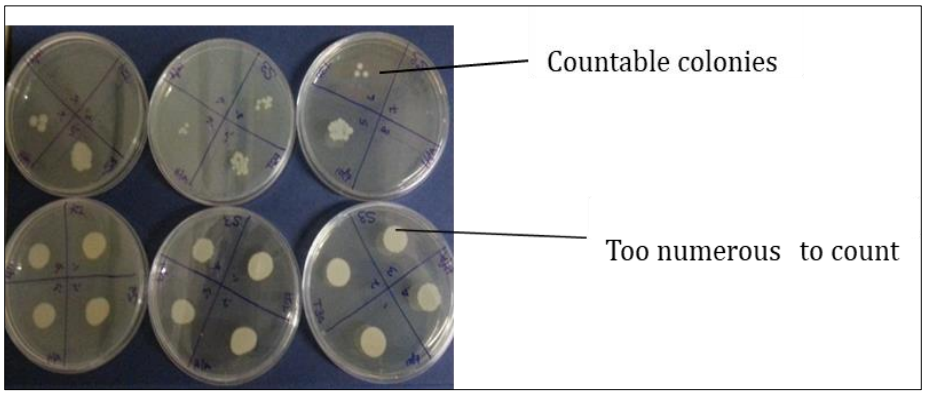

Figure 2 TSA plates used for viable count (Miles and Misra)

Viable count was conducted on the TSA plates for each dilution from the colony growth. The number of colony forming unit for each dilution was estimated from which the concentration of the undiluted sample (cfu/ml) was determined as;

2.7.1. Average number of colonies x 50 x 1/dilution factor

The values obtained were the expressed as $\log_{10}$ cfu/ml of undiluted sample. The average concentration ($\log_{10}$ cfu/ml) of the samples collected was calculated and used to plot a growth curve.

2.8. Statistical analysis

In order to obtain the standard deviation, the means and standard errors of the bacterial concentrations in the fermentation experiments conducted with respect to time using *P.oleovorans* NCIMB 6576 and *R.eutropha* NCMBI 14402, samples were analysed using Graph pad prism six software. Analysis of variance (two ways ANOVA) was used to give an analysis of the differences of the growth patterns (as means of $\log_{10}$ cfu/ml) between *P.oleovorans* NCIMB 6576 and *R.eutropha* NCMBI 14402 with respect to the oil samples.

3. Results

Two different bacteria (*P.oleovorans* NCIMB6576 and *R.eutropha* NCIMB 10442) were used for the fermentation and production of a polyhydroxyalkanoate (PHB) using TSB as the growth medium with industrial waste oil sample (PS and TS) serving as the carbon source. Results obtained from the viable count (cfu/ml) were averaged and the colony forming units ($\log_{10}$cfu/ml) determined for each experiment conducted with time. The standard deviation and standard error of the averages were analysed using graph pad prism software and the growth curves were plotted as colony forming units ($\log_{10}$.cfu/ml) against time.

3.1. PS oil sample and *P. oleovorans* NCIMB 6576

As can be seen in figure 3, the growth curve of the cultures of *P. oleovorans* NCIMB6576 grown on TSB with and without PS oil sample shows error bars in the graph for each point depicting the standard error of the mean. The initial viable count ranges between 6.37 $\log_{10}$ cfu/ml and 5.1 $\log_{10}$ cfu/ml. The viable count reached its peak after 30 hours giving approximately 9.7 $\log_{10}$ cfu/ml for *P. oleovorans* NCIMB6576 with PS oil and 9.24 $\log_{10}$ cfu/ml after 30 hours as well without the oil, showing that maximum cell count was attained at the same time. There also follows a reduction in the cell count reaching 9.0 $\log_{10}$cfu/ml for *P. oleovorans* NCIMB6576 with the oil compared to 7.08 $\log_{10}$ cfu/ml for TSB only after 48 hours.

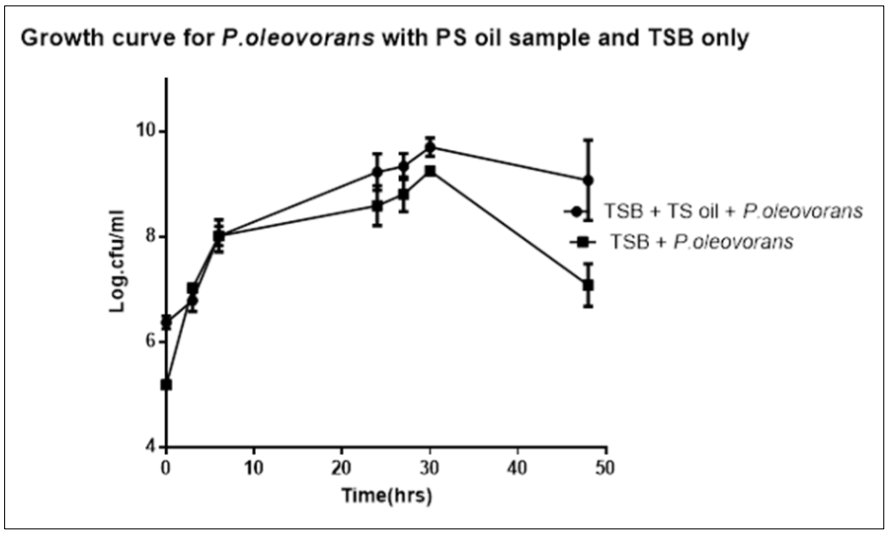

Figure 3 Growth curve of P. oleovorans NCIMB 6576 grown in tryptone soya broth with and without PS oil for 48 hours at thirty degrees Celsius and 150rpm

3.2. TS oil and *P. oleovorans* NCIMB6576

Fig 4, shows the growth curves of *P. oleovorans* NCIMB6576 grown on TSB with and without the oil sample TS, where the errors bars depicts the standard errors of the means on each point. The initial viable count at the start of the experiments shows that for *P. oleovorans* NCIMB6576 grown with the oil, there was an initial viable count of 6.1 $\log_{10}$ cfu/ml as compared to 5.1 $\log_{10}$ cfu/ml without the oil respectively. This was followed by an exponential increase in the count reaching its peak after 27 hours resulting in a 9.93 $\log_{10}$ cfu/ml for the bacteria grown with the oil and 9.2 $\log_{10}$ cfu/ml after 30 hours in the case of *P. oleovorans* NCIMB6576 grown in TSB alone. It was observe that the time at which maximum cell counts was attained is slightly longer when the oil was not used as a carbon source (30 hours) as compared to the oil control (27 hours). A decline in cell count is also noticeable after 30 hours until it reaches its minimum value of 9.4 $\log_{10}$ cfu/ml after 48 hours in the experiment involving the oil sample TS. The negative control (without oil) shows a fall in cell count of 7.0 log cfu/ml after 48 hours. This shows that, although a decline in cell count was noticeable after 48 hours in both cases, the oil control with *P. oleovorans* NCIMB6576 gave a higher cell concentration than without the oil.

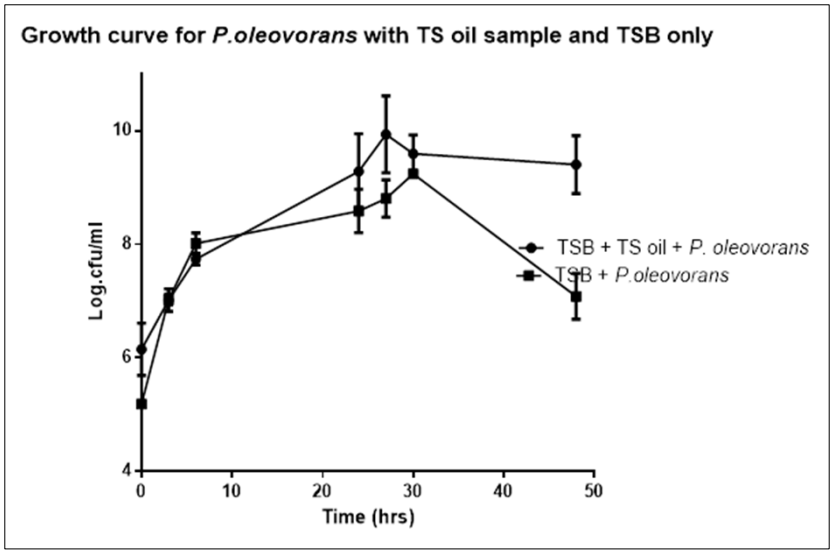

Figure 4 Growth curve of *P. oleovorans* NCIMB 6576 grown in tryptone soya broth with and without TS oil for 48 hours at thirty degrees Celsius and 150rpm

3.3. *R. eutropha* NCIMB 10442 with the oil samples PS and TS

Control experiment was carried out with *R. eutropha* NCIMB6576 as it is established in literatures that it gives appreciable yield of PHB with oils. The growth curves of this experiment (figure 5) show that the initial cell count was 3.778 $\log_{10}$ cfu/ml and 5.538 $\log_{10}$ cfu/ml respectively for PS and TS oils. The optimum viable count was attained at 27 hours with *R. eutropha* NCIMB 10442 with PS oil giving the highest count of 9.0 $\log_{10}$ cfu/ml and 8.298 $\log_{10}$ cfu/ml with TS oil. A decline in cell count was observed at 30 hours giving 8.7 $\log_{10}$ cfu/ml and 8.202 $\log_{.10}$ cfu/ml respectively further falling to 8.66 $\log_{10}$ cfu/ml and 8.23 $\log_{10}$ cfu/ml after 48 hours in both cases.

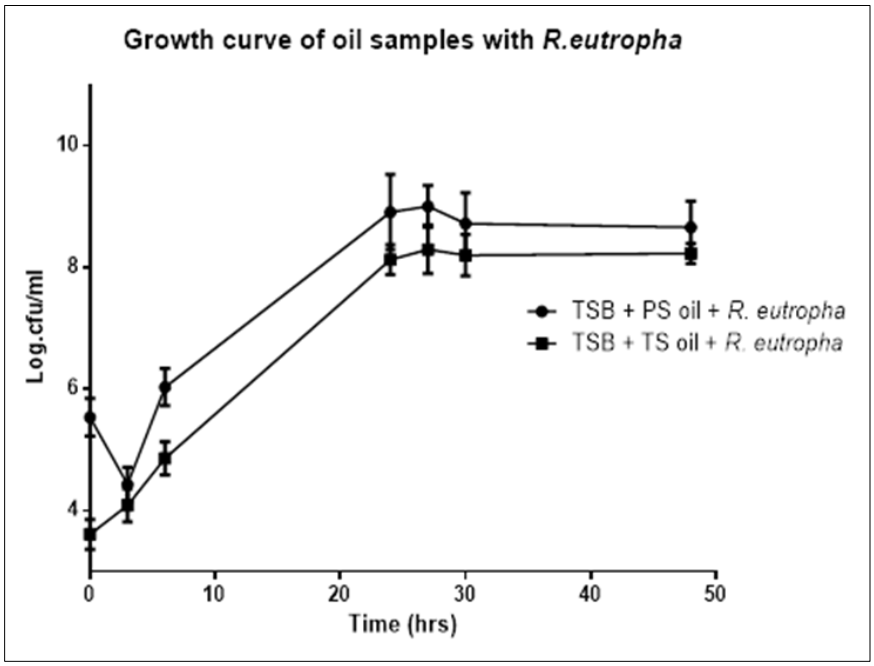

Figure 5 Growth curve of *R. eutropha* NCIMB 10442 grown in tryptone soya broth and TS oil for 48 hours at 30^{o}C and 150rpm

3.4. Comparisons between the means of the average $\log_{10}$ cfu/ml of the groups involving *P. oleovorans*

NCIMB6576 with the oil samples against each other (appendix 3a), using two way ANOVA revealed that no significant difference exists between these experimental groups involving *P. oleovorans* NCIMB6576 with the oil samples (α=0.05, p<0.001). However, significant difference was observed when the control group TSB with *P. oleovorans* NCIMB6576 was compared with groups involving *P. oleovorans* NCIMB6576 with both oil samples TS and PS. Similar result was revealed when the groups involving *R. eutropha* NCIMB10442 and the two oil samples (α=0.05, p< 0.001) were compared (appendix 3b). This shows that the pattern of growth of the bacteria is different in the oil samples signifying that the bacterial growth varies with the carbon source except in the case of *P. oleovorans* NCIMB6576 since no significant difference was realised between the groups involving the bacteria.

4. Discussion

The initial colony counts for *P. oleovorans* NCIMB 6576 varies from 6.37 $\log_{.10}$ cfu/ml to 5.1 $\log_{10}$ cfu/ml for experiment with PS oil as carbon source and TSB only. There is also a difference in the initial cell count of *P. oleovorans* NCIMB 6576 with TS oil and TSB only. Similarly, experiments with *R. eutropha* NCIMB 10442 with the oil samples also have different initial viable counts. These variations in cell counts may be due to errors attributed to improper sampling arising from improper measurements of the samples during the procedure. It is established that errors may arise during microbial procedures causing errors in preparation of the samples, formulations of the samples and media preparations and variations in microbial colony distributions (Jarvis *et al.,* 2007).

A difference in maximum viable count was noticeable after 30 hours giving 9.7 $\log_{10}$ cfu/ml in the case of *P. oleovorans* NCIMB 6576 with PS oil sample compared to 9.24 $\log_{.10}$ cfu/ml without the oil. However the time taken to attain the maximum cell count is the same for both experiments involving *P. oleovorans* NCIMB 6576 with or without the PS oil sample.

For *P. oleovorans* NCIMB 6576 with TS oil sample, the initial viable count was 6.1 log_{10}cfu/ml which varies with that without the oil which is 5.1 log_{10}cfu/ml. A high cell count of 9.93 log_{10}cfu/ml was reached after 27 hours with the oil as carbon source which differ from the peak cell count of 9.2 log_{10}cfu/ml in the negative control without the oil. This shows that the time taken to attain maximum cell count was shorter in experiment with the oil sample as carbon source (27 hours) compared to the negative control (30 hours).

A decline in cell count was noticeable in the growth patterns in both experiment involving PS and TS oil reaching its lowest after 48 hours. A count of 9.0 log_{10}cfu/ml was recorded for *P.oleovorans* NCIMB 6576 with PS oil compared to 7.08 log_{10}cfu/ml without the oil as carbon source (figure 11). Similarly, after 48 hours, *P. oleovorans* NCIMB 6576 with TS oil gave a declined count of 9.4 log_{10}cfu/ml compared to 7.0 log_{10}cfu/ml without the oil. This shows that both oil samples have a positive influence on the bacterial growth since more counts were realised after 48 hours in the oil controls compared to the negative control without oil. A number of researches have supported the fact that oil composition has a significant influence on PHA production by enhancing bacterial growth rates and PHA production as a result of frying and cooking which adds certain food substances that influence bacterial growth and polymer production (Obruca *et al.,* 2010, Verlinden *et al.,* 2011). As the oil samples used are waste cooking oils, it is likely that cooking has caused some changes in the oil composition as a result of incorporation of food substances like proteins and carbohydrates besides the change in fatty acid contents.

The *R. eutropha* NCIMB 10442 control experiments with the oils revealed an initial viable count of 5.538 log_{10} cfu/ml and 3.778 log_{10} cfu/ml with PS and TS oil samples respectively. The highest viable count of 9.0 log_{10} cfu/ml and 8.7 log_{10} cfu/ml was reached after 30 hours respectively. This is followed by a decline in cell count after 48 hours leading to 8.66 log_{10} cfu/ml and 8.23 log_{10} cfu/ml in both cases.

5. Conclusion

The study investigate the use of waste oil in the production of bioplastic (polyhydroalkanoate) using the techniques of microbial biotechnology. Pseudomonas oleovorans was used as the microorganism and simple static and agitated fermentation was used to produce highly efficient and productive bioplastic within the period of 20days. This study demonstrated the act of cost-effective and environmental friendly techniques in biotechnology which remains a tool for the countries econonmic growth and development. Bioplastic has recently attracted interest in the field of science as the application of the product continue to excel for national growth and development. It has found application in textile industries, cars, rubber, etc. Discovering a cost effective approach to the production of this value added product is a step forward in the area of biotechnology

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare no conflict of interest in the work, however, traces of constructive criticism was observed at the cost of data collection but eventually, mutual agreement was mate at end and result submitted is a product of duely accepted process by all members of the team.

Statement of ethical approval

All protocols involving ethical standard has been followed duely. Permission for data collection and approval for risks instances were strictly taken into consideration before any practical work was observed. Experience laboratory attendant were made available and strict were followed accordingly.

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