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Production and Optimization of Biofuel from Lignocellulosic waste using natural Bacterial consortia

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ABSTRACT: Biofuels are produced from microorganisms as a by product of metabolic activity of organic or cellulosic waste products. The target was achieved the low cast renewable source to fulfil the current requirement. In order to be considered a bio fuel the fuel must contain over 80% renewable materials. Ethanol is and highly demand bio fuel made from feed stocks such as different food crops and food crop wastes after the pre-treatment. We explore such the bacterial consortium that couples the high cellulosic activity of the mixed culture of bacteria to ferment hexose and pentose sugars to ethanol. The present study we analysed the three bacterial consortia for the degradation of the agro cellulosic waste such as turmeric stem, bamboo leaf, orange peel and Jack fruit inner peel by submerged fermentation for cellulose production. In quantification of biofuel, bacterial species *Enterobacter* sp (SA-02), was most efficient and produced maximum amount of bio ethanol on the sixth day of incubation it showed 2.04g/l ethanol. Further findings showed that Bacillus sp (SA-05), Pseudomonas sp (SA-07) played on efficient role in 5.60 g/l and 4.03 g/l respectively and bacterial consortia showed the maximum level of enzyme activities and pattern of ethanol production. The optimization study for ethanol production makes clear to the fact that high temperature and pH, only 35^0c and pH 8 were considered optimum. At the optimum substrate concentration was found to be 10.25 g/l after 6 days of incubation which summed as a total yield of 0.132 g of ethanol per g of dried Jack fruit inner peel. This current study evaluates the potential of turmeric stem, bamboo leaf, orange peel and Jack fruit inner peel as a production. These existing studies evaluate the potential jack fruit inner peel as a probable substrate for ethanol production. We found that Natural bacterial consortia showed the efficient production is required to make the process industrially feasible.

Keywords: Ethanol, bacterial consortia, fermentation, optimization, Enzyme activity.

INTRODUCTION

Bioenergy is an Accepted significant contribution in atmospheric change improvement strategies, particularly for electric power, liquid fuel, biochemical purposes. The traditional bio energy requires complex supply chains; the conversion of biomass is a pre requirement for the large-scale exploitation of bio energy (Rose *et al*., 2014). Bio ethanol is being widely considered as a renewable fuel source because in many respects its superior to gasoline fuel. Ethanol provides energy that is renewable and carbon intensive than oil. It is a bio-fuel formed from living things through biochemical reactions. An efficient ethanol production requires four components: fermentable carbohydrates, an efficient yeast strain, a few nutrients and simple culture conditions.

Lignocellulose is the most prevalent and plentiful resource of organic carbon and it is a wide choice of biomass for bio-fuel production, and it has reimbursement in agriculture, environment renewable power development and national production (Lynd *et al*., 2021). Though the main technological obstruction to more extensive consumption of lignocelluloses for ethanol production has been the deficient of low cast technologies to overcome the rebellion of its chemical structure, which is composed of closely intertwined cellulose, hemicelluloses, and lignin (Kalyani *et al*., 2013; Zuroff *et al*., 2019).

However, the use of food crops and grains as feed stocks eventually leads to scarcity of food and also increase the prices of crops. The use of such food crops involves huge amounts of arable and fertile soil along with human labour which increases the overall production cost. To overcome the challenges of using food crops in industrial scale bioethanol production, different lignocellulosic biomass, forestry products, energy crops, waste crops, and solid wastes like rice straw, corn straw, wheat straw, grasses, sawdust and wood chips are being used as substrates. However, they involved chemical pre-treatment, energy consumption, carbon emission and high cost (Elshahed *et al.*, 2010).

Nowadays, Fruit and vegetable wastes like date palm leaves, Mango wastes, orange wastes, oil palm empty fruit wastes are used as substrates for ethanol production these wastes are generated world-wide and

also contain rich source of fermentable sugars to help the production of ethanol and also support the reduction of agro wastes (Kim et al., 2015). Among others Turmeric, Orange, bamboo and jack fruit is grown abundantly in Tamilnadu and India is the highest producing country and we can get the substrates for ethanol production with10 thousand tons per year. Meanwhile the impact of seasonal variations on the cultivation is almost minimal and there is no significant changes observed in nutritional composition, its readily available for continuous supply as a substrate to support the industrial bio ethanol production.

Natural bacterial consortia are innately capable of extensive conversion of lignocellulostic biomass (Wongwilaiwalin *et al*., 2012). In addition natural bacterial consortia offer other advantages, such as the ability to use a wide variety of natural lignocellulostic biomass substrates (Feng *et al*., 2011 and Lv *et al*., 2013), exceptional self-stability and few functional needs such as pre-treatment or sterilization (Hui *et al*., 2013 and Guo *et al*., 2015). Still it remains a challenge to decipher and optimize cellulosic ethanol production by natural bacteria consortia, because natural bacteria consortia are complex harbour multiple populations with overlapping niches formed by various uncultured and cultured bacteria with or without cellulolytic activities, thus generally resulting in poor ethanol production (Haruta *et al*., 2020).

Here we found that non- cellulolytic microbes play essential role in recovering the cellulose fermentation concert of natural bacterial consortia, and generate an well-organized way to develop ethanol manufacture of innate bacterial consortia.

In this research, we have future an association of bacterial consisting of the cellulolytic bacteria such as pseudomonas sp., bacillus sp., Enterobacter sp., that the fermentation steps are association can be used on a fermentable substrate with a stable grouping for the manufacture of bio ethanol.

MATERIAL AND METHODS

Collection of the agricultural biomass for bacterial isolation. The agro waste used in this study was turmeric stem, bamboo leaf, orange peel and Jack fruit inner peel were washed and dried at atmospheric temperature (28 ±2° C) for 4 days. The dry Agro wastes was further ground in electric blender, filtered with a Mesh (0.250) sieve and stored under dry place.

Waste Substrate sample preparation

Collected substrate was air dried ground and conceded through a 2mm pore size sieve. 10g of waste substrate was dissolved in 1000 ml of deionised water, shaken strongly and filtered through whatman no 1 filter paper. Filtrate is used for further analysis.

Estimation of crude fibre collected sample

1.5 g of crude sample was weighed in a glass container, noted (w) and the container fixed in to the digestion tubes. Then 1.25 % sulphuric acid (200 ml) was added and heated at 400°C for 30 min. The containers were cooled and acid solution was filtered for deposits, it washed twice in hot distilled water. The same procedure was repeated with 200 ml of 1.25% of sodium hydroxide. The container was removed and kept for drying in hot air oven at 100°C for 1 hour and cooled in desiccators (Hedge *et al*., 2013). The cooled container were (W1) and kept for ashing 54± 5°C for 4 h. the results were expressed in percentage for crude fibre as follows.

Estimation of Carbohydrates. Carbohydrates content was measured by Anthrone method (Hedge *et al*., 2013). 100 mg of the Skin Material of agro waste was taken into a boiling tube. The tube was added with 5 ml of 2.5 N-Hcl and kept it in boiling water bath for three hours and cooled to room temperature. Then neutralized the sample with solid sodium carbonate, and made up to 100 ml and centrifuged the sample. The supernatant was collected and made up to 100 ml and centrifuged the sample. The supernatant was collected and 4ml of anthrone reagent was added. The tube was kept in boiling water bath. Optical density was measured at 630 nm (Ezebuiro *et al*., 2015).

Isolation of bacteria. Serial dilution agar plate method used for the isolation and enumeration of bacteria which are the most prevalent microorganisms. The sample was serially diluted up to 10-7 and 1 ml of sample suspension were taken from 10^{-2} to 10^{-7} and pipette out into sterile Petri plate and nutrient agar medium was mixed with sample by gentle rotation. The plates were allowed to solidify and incubated (Rahman *et al*., 2012).

Preparation of Bacterial consortia. For the preparation of the natural bacterial consortium, the colonies were plated on minimal agar medium supplemented with carboxy methyl cellulose (CMC) and incubated at 37°C. After 12 hours, the strains at log phase were inoculated on 250 ml flasks containing 100 ml nutrient broth and incubated at 37°C at 200 rpm for 12 hours. 5 ml from each broth was added to a sterile test tube and centrifuged at 5000 rpm for 15 minutes. The supernatant was discarded and normal saline solution was added to the pellets and vortexes well. A volume of 0.1 ml every colony was added to nutrient broth and incubated overnight. This prepared consortium was used to inoculate for the ethanol production. This study to develop successful bacteria consortium that can concomitantly degrade the different substrates with the help of their cellulase enzymes in short span of time under natural conditions without producing foul odour. Effective bacterial consortia for the degradation of orange peel, bamboo leaves and taioca shell was prepared as per method (Tiwari *et al*., 2015). Preparation of successful microbial consortium, the bacterial cultures should be suitable with each other in order to produce all enzymes required for the degradation of these substrates. The compatibility of the consortia was checked by cross streaking method in Nutrient agar plate.

Inoculum Preparation. The selected isolates which are non antagonistic against each other were selected for testing their suitability for preparing the consortium of microorganisms to be used for improving the speed of utilize the nutrients. The selection was based on growth rate and the efficiency of the isolate to produce specific

enzymes which are having definite role in bioethanol production (Mandal *et al*., 2012).

Inoculum medium was prepared in 250 ml conical flask with a working volume of 100 ml Nutrient agar medium containing (g/l) peptone 0.5 g beef extract 0.3 g, sodium chloride 0.5 g and distilled water 100 ml. the medium was sterilized at 121°C for 15 minutes. One loop of isolated bacterial strains from one day old culture on Nutrient agar slant was transferred to 250 ml. Clonical flask having 100 ml nutrient broth. The flask was incubated at 37^0C and 150 rpm in an incubator shaker for 24 hours.

Fermentation

The fermentation process was carried out in I litre flask contains 500ml of fermentation medium maintained at 250 rpm in rotary shaker using pre treated orange peel powder, bamboo leaves powder and turmeric stem powder were used as raw material and supplemented with the following composition $NaNo_3$ – 3g, $MgSo_4$ 7 H_2O – 0.5 g, K_2HPO_4 – 1.0 g $FeSo_4$&H_2O- 0.1 g P^H – 7 ± 2. The media used for fermentation are separately. The following treatments were maintained to study the impact of selected three substrates in bringing the ethanol production. The Flask was inoculated with 24 hours culture. The inoculum development and fermentation were carried out at 37± 1^0C in a orbital shaker at 250 rpm. After fermentation the bio -ethanol was extracted by standard distillation method (Adarsha *et al*., 2016).

Estimation of ethanol. 10 ml of extract in a glass beaker containing 25 ml of 3.4 % chromic acid 33.768 g of potassium dichromate is liquefied in 350 ml of purified water in standard measuring flask and the flask is kept in an ice bath and slowly added 350 ml of concentrated sulphuric acid. The content is made up to 1000 ml with distilled water) and added 50ml of double distilled water then contents were mixed thoroughly and then it was heated up to 80°C for 15 min. The absorbance was recorded in a spectrophotometer at 580 nm. The concentration of alcohol content was plotted against respective absorbance values at 580 nm to draw ethanol standard curve (Gao *et al*., 2014).

Optimization of pH. 100ml of jack fruit peel was prepared and dispensed into different conical flasks. The pH of the decontaminated broth was customary as 3, 4, 5, 6 and 7 in a conical flask. A loopful of Natural bacterium consortia was inoculated into all the flasks. The flasks were incubated at 28°C for 5days. After incubation the percentage of ethanol production was determined (Du *et al*., 2014).

Optimization of Temperature. 100 ml of jack fruit peel was prepared and distributed into diverse conical flasks. The temperature of the sterilized broth was set as 20°C, 25°C, 30°C, 35°C and 40°C in a flask. A loopful of natural bacterium consortia was inoculated into the flasks and incubated for 5 days. After incubation, the percentage of ethanol production was determined. Optimization of yeast concentration for ethanol production (Li *et al*., 2013).

The optimum quantity of jack fruit peel solution was taken in fermentation flask and the pH and temperature were maintained at 5 and 35^0C. Numerous amounts of yeast like 1, 2, 4 and 8ml were added and reserved for a period of 5 days and the fermented results were analysed.

Cell Biomass. The dried peel of the sample was assessed by centrifuging the identified volume of sample in a pre dried and pre weighed centrifuge tube for 20 minutes. After receiving cell suspension in 2ml of distilled water and further centrifugation, the cell suspension was dried. The dried cell suspension was calculated by reweighing the tube (Pei *et al.*, 2012).

RESULT AND DISCUSSION

In the current study, we have confirmed the total carbohydrate content of the turmeric stem, orange peel, bamboo leaves, jack fruit inner peel were before and after the pre-treatment (Table 1).This result indicated that the total availability of carbohydrate before before and after pre-treatment was 73.8±2.6 and 23.6 ± 0.3% (w/w), respectively. The best pre-treatment method was Acid pre-treatment method that increase the carbohydrate content of substrates.

Table 1: Carbohydrate content of Agro substrates.

Agro substrates	Total carbohydrate (% dry weight)
acid pre treated Turmeric stem before	56.2±1.6
acid pretreated Turmeric stem after	10.3± 0.5
acid pretreated Orange peel before	73.8±2.6
acid pretreated Orange peel after	23.6 ± 0.3%
Acid pretreated bamboo leaves before	49.3 ±1.2
Acid pretreated bamboo leaves after	18.6± 0.3
Acid pretreated jack fruit inner peel before	50.4±2.1
Acid pretreated jack fruit inner peel after	20.7± 3.4

We explore three different bacterial consortia used in various agro wastes like orange peel, bamboo leaves and jack fruit inner peel are exhibited high ethanol production. All three selected bacterial species like Enterobacter (SA), Bacillus (SA), Pseudomonas (SA) were isolated from substrate and inoculated into medium containing natural substrates like orange peel, bamboo leaves, Turmeric stem and jack fruit inner peel for ethanol production by fermentation process. Bio ethanol was formed by fermentation and purified by distillation unit and the quantity of ethanol was intended using specific gravity method. The content of bio ethanol was confirmed by measuring specific gravity of distillate (Hasunuma *et al*., 2013).

Table 2: Production of ethanol by using individual bacteria and bacterial consortium.

Sr. No.	Sample	Strain Name	Ethanol production on Sixth day
1.	Turmeric stem	Enterobacter sp (SA-02) - I	2.04 G/L
2.	Orange peel	Bacillus sp (SA-05)- II	5.60 G/L
3.	Jack fruit inner peel	Pseudomonas sp (SA-07) - III	4.03 G/L
4.	Jack fruit inner peel as substrate	Bacterial consortium I,II and III	10.25± 0.34 G/L

All three bacteria were inoculated in turmeric stem, orange peel, bamboo leaves and jack fruit inner peel as substrate, and incubated at 37°C was also observed. In quantification of bio ethanol, bacterial species Enterobacter sp (SA-02) was showed effective and maximum amount of ethanol on the sixth day of incubation, it exhibited 2.04g/l ethanol (Gao *et al*., 2014). Further findings showed that Bacillus sp (SA-05), Pseudomonas sp (SA-07) played on efficient role in 5.60 g/l and 4.03 g/l ethanol respectively these strains successfully boosted the ethanol production when compared with other bacterial consortia (Table 2). By using all three bacteria we form new bacterial consortium to achieve the highest ethanol production. Our natural bacterial consortia showed the maximum level ethanol production up to 10.25± 0.34 (g/l).

Table 3: Effect of Substrate on ethanol production by using bacterial consortium (I, II and III).

Sr. No.	Substrate	Ethanol production
1.	Turmeric stem	3.67 ± 0.23 G/L
2.	Orange peel	7.43± 0.34 G/L
3.	Bamboo leaves	4.46± 0.54 G/L
4.	Jack fruit inner peel	10.25± 0.34 G/L

This research was carried out to produce bio ethanol from orange peel, turmeric stem, bamboo leaves and jack fruit inner peel are the cellulose waste served as substrate for the fermentation process, cellulose can be converted to bio ethanol by following pre-treatment, hydrolysis and fermentation by appropriate Bacterial consortium (Sarkar *et al*., 2012). The ethanol production by turmeric stem 3.67 ± 0.23, orange peel 7.43± 0.34, bamboo leaves 4.46± 0.54 and jack fruit 10.25± 0.34 (g/l) Among four jack fruit inner peel shows maximum production (Table 3).

Temperature was a chief restraint that regulates the Ethanol production. To identify the optimum temperature for bio ethanol fermentation, the solutions were kept at 25, 30, 35 and 40°C .Two parameters are concurrently studied, the growth of Bacterial consortium and optimum temperature.

The samples were taken from 12 hours incubation and fermentation was carried out for up to 48 hours. The result shown at 30°C ethanol yield was maximum and turned out to be 2.02 G/L. Though increasing temperature more than 30^0c the growth of bacterial consortium as well as production of alcohol also decreased. This decrease pronounced at 40°C so 33°C was selected as optimum temperature for ethanol production.

Table 4: Effect of Temperature on Bacterial consortium growth and ethanol Production.

Time	Bacterial Growth (In OD)				Ethanol Production(In G/L)			
	25°C	30°C	35°C	40°C	25°C	30°C	35°C	40°C
0	0.22	0.24	0.26	0.23	0	0	0	0
12	0.44	0.70	0.61	0.46	1.2	4.1	2.0	1
24	0.92	1.42	1.28	0.74	3	7.6	5.1	2.2
36	1.30	1.45	1.58	0.98	4.5	8.5	6.4	2.6
48	1.75	2.02	1.91	1.20	5.6	10.5	8.2	3

The temperature tolerance will vary based on the sugar concentration of the medium, sometimes the for fermentation of molasses at 35°C was possible when the sugar concentration was 20 % (Janani *et al*., 2022).

After the confirmation of optimum temperature 30°C was selected for further studies and subjected to P^H treatments 5, 6, 7 and 8. The findings are at P^H 5 the fermentation took place but ethanol production was low. Finest results were found that at P^H 6 where maximum ethanol production was noticed. Similarly, (Manmai *et a.,l* 2020; Hemamalini *et al*., 2023) also found that an increased alcohol production and optimum growth also increase in P^H range between 4.0-5.0. Founded on fermentation competence the P^H 6 was designated for added investigation.

Table 5: Effect of pH on Bacterial consortium growth and ethanol Production.

Time	Bacterial Growth (In OD)				Ethanol Production (In G/L)			
	P^H 5	P^H 6	P^H 7	P^H 8	P^H 5	P^H 6	P^H 7	P^H 8
0	0.22	0.22	0.26	0.23	0	0	0	0
12	0.47	0.86	0.78	0.86	2	3.4	2.4	3.4
24	0.97	1.56	1.32	1.56	3	7.6	5.1	2.2
36	1.30	2.1	1.97	2.1	3.7	7.5	4.4	6.4
48	1.57	2.26	2.21	2.50	7.6	11.5	8.1	9.6

After optimizing the numerous limits like P^H, temperature, substrate concentration, etc. the experimentation was ascended up from conical flask to fermented. The optimum of our finding was take that is substrate concentration of 10.25± 0.34 G/L, P^H 6 and the temperature 30 °C and incubation period 48 Hrs to further continuation of the experiment on fermentation. Fermentors are designed to provide best possible growth and biosynthesis circumstances for industrially important microbes and natural bacterial consortium. In fermentor, it's easier to control various parameters like P^H, temperature, sugar concentration of obtaining the correct product such as ethanol.

CONCLUSIONS

Bio ethanol is one of the most suitable and cost effective bio fuel. In our current finding we gained bio ethanol from four orange peel, turmeric stem, bamboo leaves and jack fruit inner peel collected from local market around Trichy. With a isolation of bacterial consortium containing Enterobacter sp (SA-02), Bacillus sp (SA-05) and Pseudomonas sp (SA-07), the ethanol was produced by fermentation process. After 10 days we could gained10.25± 0.34 G/L ethanol from jack fruit inner peel as a best substrate and maintaining P^H of 6 and of 30°C. We could infer that more concentrated form of ethanol could be obtained by distillation. The more concentrated ethanol could be used as a bio fuel, which releases no toxic gases out in the environment. As a result, the findings of this study show cases the effects of different pre-treatment, processing and optimization methods for the production of bio ethanol from variety of agro wastes.

FUTURE SCOPE

The Agro-wastes used in this study are reusable and renewable sources which is used for energy conversion gave as Cost effective, alternative energy source and create eco friendly, pollution free environments.

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Conflict of Interest. None.

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