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Bioethanol Production Using Bacterial Consortia From Waste Cellulosic Waste

***Mrs. T.* VINOTHA**
Assistant Professor
Department of Microbiology
Hindusthan College of Arts and Science
Coimbatore, Tamilnadu
E-mail ID: Vinots2014@gmail.com

***Dr. N.* UMAMAHESWARI**
Assistant Professor & Head
Department of Life Science
S.T.E.T women's College,
Sundarakkottai, Mannargudi, Tamilnadu
E-mail ID: umasamyamf@gmail.com

ABSTRACT

One of the important targets of industrial biotechnology is using cheap biomass resources. The traditional strategy is microbial fermentations with single strain. We explored such a consortium that couples the high cellulolytic activity of the mixed culture of bacteria to ferment hexose and pentose sugars to ethanol. Consortium stability was demonstrated by culturing the three microbes on a mixture of cellulose, as a first step towards understanding and manipulating this consortium, we developed a simple dynamic model with unstructured descriptions of enzyme synthesis, cellulose and hemicellulose degradation, sugar uptake, cell growth, and ethanol production. The present study aims to the use of the bacterial consortia for the degradation of the cellulosic waste by submerged fermentation for cellulase production. The Three bacterial strains were tested to find their ability to produce cellulases, which catalyze the degradation of cellulose, which is a linear polymer made of glucose subunits linked by β-1, 4 glycosidic bonds. All three bacterial strains was noticed to show maximum zone of hydrolysis of carboxy-methyl cellulose and four bacterial mixture (consotia) produce higher activities of the cellulases were determined by Carboxy-methly cellulase assay (CMCase). In quantification of bioethanol, bacterial species ***Enterobactor cloaca*** was most efficient and produced maximum amount of bioethanol on the fifth day of incubation, it showed 2.06 g/L ethanol titer. Further studies showed that ***Pseudomonas aeroginosa, Bacillus clausii,*** played an important role in the high ethanol productivity upto 8.80 ± 0.75 **(G/L) &** 9.03 ± 0.08 **(G/L)** respectively. And Bacterial consortia have exhibited high level of enzyme activities and pattern of ethanol production as 10.91 ± 0.49 **(G/L)** compared to single strain. Maximum specific activities of enzymes were obtained between 15 to 25 days of culture growth.

Keywords: Fruit peel waste, microbial consortia, cellulosic enzyme, submerged Fermentation

Introduction:

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Bioethanol is alternative energy sources which are production from agricultural waste and it is a promising technology, the process has several challenges and limitations they are transportation of biomass, handling, pretreatmen methods for total delignification of lignocellulosics and appropriate fermentative organism **(Sarkar et al. 2012).**

Traditionally, ethanol have been produced from the starch based utilizing enzymatic process lie saccharification; so it leads to the production of a glucose, then it was fermented to ethanol by *Saccharomyces sp*. Cellulose is a homopolymer with more than 3000 repeated glucose units consisting of β (1-4 bonds) **(Sarkar et al. 2012).** Cellulose is being assembled from glucose produced in the living plant cell through photosynthesis with about 7.5×1010 tons being synthesized annually. The composition of these three components varies from one plant species to other. Agricultural biomass constitutes about 10-25% lignin content, 20-50% have hemicellulose and cellulose. **(Xu et al. 1998; Talebnia et al. 2010)**

Conversion Technologies for Ethanol Production

Different conversion technologies are available for conversion of cellulosic feedstock into ethanol. Two main technologies commonly used for cellulose conversion are hydrolysis and thermochemical conversion. Hydrolysis is carried out in two stages. Pre-treatment of biomass is the first stage and enzymatic hydrolysis is the second stage for glucose conversion.

Biomass Pre-treatment

Pre-treatment is carried out in different ways which mainly includes physical, chemical, biological and combined processes. Pre-treatment is a process which converts the carbohydrates in an inactive native form to active accessible forms for enzymatic hydrolysis **(Lynd *et al.*, 2002).** Commonly used techniques for feedstock pre-treatment are thermal (steam, steam explosion, liquid hot water), Ammonia Fibre Expansion (AFE), acid, alkaline, oxidative and organo solvent. Depolymerisation of lignin is usually carried out by the thermochemical method due to its high complexity. Thermal pre-treatment requires high operating conditions such as high pressure and high temperature (up to 200°C). Acid and alkaline pre-treatment are normally carried out in industries as they efficiently break down the lignin products at a high concentration. The disadvantages in the chemical pre-treatment methods are corrosion of the operating vessel and formation of recalcitrant toxic chemicals in the effluents which cause

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environmental pollution. To promise environmental safety and low operating cost for bioethanol production, biological pre-treatment of biomass is appreciated.

Cellulase

Cellulose is an enzyme which is produced by fungi and bacteria that are able to degrade cellulose into cellulases. Cellulases have breakdown the cellulose molecule into monosaccharides and polysaccharides such as shorter polysaccharides and oligosaccharides. Cellulose breakdown has a major constituent of plants are available for consumption and use in chemical reactions. The reaction involved is the hydrolysis of the 1, 4-beta-D-glycosidic linkages in cellulose, hemicelluloses, lichenin and cereal beta-D-glucans. Because cellulose molecules bind strongly with each other and cellulose is relatively difficult compared to the breakdown of other polysaccharides. **(Bhat, M.K ., 1997)**

In this research, we have proposed a consortium of bacterium consisting of the cellulolytic bacterias such as ***Pseudomonas aeroginosa, Bacillus clausii, and Enterobactor cloaca*** that the fermentation steps are consolidated into a single reactor. The goal of the present study is to demonstrate consortium can be used on a fermentable substrate with a stable combination for the production of bioethanol.

Material & Methods

Collection of the biomass

The agricultural biomass used in this study was Orange peel, Bamboo leaves, Tapioca shell. The Orange peel, Bamboo leaves, Tapioca shell were washed and dried at atmospheric temperature (28 ± 2 °C) for 3 days. The dry biomass was further ground in electric blender, filtered with a Mesh (0.250 mm) sieve and stored under dry place.

Acid pre-treatment (**Sharma et al. (2007)**

The dilute sulfuric acid pre-treatment of fruit waste substrate was carried out using acid concentration (1-5% w/v) and incubation time (15-60 min) at 121°C. The hydrolysate was separated by filtering the contents through muslin cloth. The residual biomass was washed with tap water till the neutral pH and dried in a hot air oven at 60°C.

PHYSICOCHEMICAL CHARACTERISTICS

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Waste substrate sample preparation

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

Estimation of Crude fibre

Collected Sample (1gm) was weighed in a glass crucible, noted (W) and the crucible fixed into the digestion tubes. Then 1.25% sulphuric acid (200 ml) was added and heated at 400°C for 30 min. The crucibles were cooled and acid solution was filtered for residues, it washed thrice in hot distilled water. The same procedure was repeated with 200 ml of 1.25% of sodium hydroxide. The crucibles were removed and kept for drying in hot air oven at 100°C for 1 h and cooled in a desiccator. **(Hedge and Hofreiter, 1962).** The cooled crucibles were weighed (W_1) and kept for ashing 55 ± 5°C for 4 h. The results were expressed in percentage for crude fibre as follows

$$\text{Crude fibre (\%)} = \frac{W_1 - W_2}{w} \times 100$$

Estimation of Carbohydrate

Carbohydrates content was measured by Anthrone method (Hedge and Hofreiter, 1962). 100 mg of the Tissue 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 one ml with distilled water, and 4ml of anthrone reagent was added. The tube was kept in boiling water bath. Optical density was measured at 630 nm. **(Hedge and Hofreiter, 1962)**

$$\text{Carbhohydrate(mg/g)} = \frac{\text{mg of glucose}}{\text{Volume of test sample}} \times 100$$

Isolation of bacteria (Norris and Ribbons, 1972)

Serial dilution agar plate method used for the isolation and enumeration of bacteria which are the most prevalent microorganisms. The sample was serially diluted upto 10^{-7} and 1 ml of sample suspension were taken from 10^{-2} to 10^{-7} and pipette out into sterile petriplate and nutrient agar medium was mixed with sample by gentle rotation. The plates were allowed to solidify and incubated**. (Apun et al. 2000)**

Production of Bio-Ethanol

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Preparation of Bacterial Consortia

For the preparation of the bacterial consortium, the colonies were plated on minimal agar medium supplemented with carboxy methyl cellulose (CMC) and incubated at 37 ºC. After 12 hours, the strains at log phase were inoculated on 250 mL flasks containing 100 ml nutrient broth and incubated at 37 ºC 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 of each colony was added to nutrient broth and incubated overnight. This prepared consortium was used to inoculate for the ethanol production.

This study is to develop successful bacterial 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 organe peel, bamboo leaves and tapioca shell was prepared as per method proposed by **(Rahman, 2002).** Prepertation 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.

Cross streak method

The antagonistic activity of cellulase producing isolates was performed by using the cross streak method **(Shirling E.B & Gottlieb. D., 1966).** Nutrient agar plates were prepared and inoculated with one isolates by a single streak in the center of the petri plate and test organisms by a single streak at 90º angles. The streak plates were incubated at 38ºC for 24 Hrs. Antagonism was observed by the inhibition of the test organism.

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 enhancing 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. **(Apun et al. 2000)**

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Inoculum medium was prepared in 250 mL Erlenmeyer flask with a working volume of 100 mL Nutrient agar medium containing (g/1) 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 Erlenmeyer flask having 100 mL Nutrient broth. The flask was incubated at 37°C and 150 rpm in an incubator shaker for 24 hours.

Fermentation

The fermentation process was carried out in 500 ml litre flasks maintained at 250rpm in rotary shaker using pre-treated Orange peel powder, bamboo leaves powder and tapioca shell powder were used as raw material and supplemented with the following composition

$NaNO_3$	-	3 g
$MgSo_47H_2O$	-	0.5g
Kcl	-	0.5g
K_2HPO_4	-	1.0g
$FeSo_4\ 7H_2O$	-	0.1g
pH	-	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 flasks were inoculated with 24 hours culture. The inoculam development and fermentation were carried out at 37±1ºC in a orbital shaker at 250 rpm. After fermentation the bio-ethanol was extracted by standard distillation method **(Caputi *et al.*, 1968).**

Screening for ethanol

10ml of fermented sample

↓

Pinch a potassium dichromate and a few drop of H_2SO_4 were added.

↓

Colour of the sample turns from pink to green indicates the presence of bio-ethanol.

Estimation of ethanol

10 ml of distillate in a beaker containing 25 ml of 3.4 % chromic acid (33.768 g of potassium dichromate is dissolved in 350 ml of distilled water in standard measuring flask and the flask is kept in an ice bath and slowly added 350 ml of concentrated sulphuric acid. The

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content is made upto 1000ml with distilled water) and added 50ml of double distilled water then contents were mixed thoroughly and then it was heated upto 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 **(Caputi *et al.*, 1968).**

Estimation of bioethanol

Qualitative estimation

Bioethanol production was examined by Jones reagent **(Jones 1953).** One milliliter of K2Cr2O7 (2 %), 5 ml of H2SO4 (concentrated) and 3 ml of sample were added to Jones reagent. Ethanol was oxidized into acetic acid with potassium dichromate in the presence of sulfuric acid and gave blue-green color. Green color indicates positive test (Caputi et al. 1968). Quantitative estimation Substrate solution was distilled in alcohol distillation unit for quantitative estimation of bioethanol.

Quantity estimation bioethanol

Twenty-five milliliters of fermented sample was mixed with 150 ml of distilled water, and distillation was performed; 90 ml of distillate was collected, 100 ml of distilled water was added, and the resulting mixture was poured to 25-ml specific gravity bottle **(Pharmacopoeia of India 1985).**

Statistical analysis:

The results obtained in the study were compared from one-way analysis of variance (one-way ANOVA) and multiple range tests to find the differences between the measurement means at 5 % (0.05) significance level. The data were analysed using IBM® SPSS® Statistics Version 20.0 **(Ezebuiro et al. 2015).**

Result And Discussion:

In the present study, we have find out that the total carbohydrate composition of the Orange peel, bamboo leaves and tapioca shell were before and after pre-treated is shown in Table 1. This result indicated that the total available carbohydrate before and after pre-treatment were 75.8 ± 2.9 and 25.9 ± 0.2 % (w/w), respectively. The best pre-treatment method developed in acid pre-treatment that increase in total carbohydrate of 95.7 ± 1.55 % dry weight. Finally we analyzed 3 different natural bacterial consortia from a diferent substrate like Orange peel, Bamboo leaves, and Tapioca shell have exhibited relatively high ethanol production. All three

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selected bacterial species like ***Pseudomonas aeroginosa, Bacillus clausii, and Enterobactor cloaca*** were isolated from this substrate and inoculated into Orange peel for bioethanol production by the process of fermentation. Bioethanol was produced by fermentation and distilled by distillation unit, and the amount of ethanol was calculated using specific gravity method. The bioethanol content was determined by measuring specific gravity of the distillate according to the procedure was described by **(Amerine and Ough., 1984).**

All three bacteria were inoculated in Orange peel substrate, and incubated at 37°C was also observed. In quantification of bioethanol, bacterial species ***Enterobactor cloaca*** was most efficient and produced maximum amount of bioethanol on the fifth day of incubation, it showed 2.06 g/L ethanol titre. Further studies showed that ***Pseudomonas aeroginosa, Bacillus clausii,*** played an important role in the high ethanol productivity up to 8.80 ± 0.75 **(G/L) &** 9.03 ± 0.08 **(G/L)** respectively and these strain effectively boosted the ethanol production of various other natural bacterial consortia. Finally, we developed a new consortium, to achieve the highest ethanol production reported for natural consortia. The ethanol conversion ratio reached 88%, with ethanol titres up to 10.91 ± 0.49 **(G/L)**

This study was carried out to produce bioethanol from Orange peel, bamboo leaves and tapioca shell are the cellulose waste using bacterial isolates from agro-waste- These served as substrates for the bacterial cultures used in the fermentation process, cellulose can be converted to bioethanol by following pre-treatment, hydrolysis and fermentation by appropriate microorganisms **(Sarkar et al. 2012).**

Pre-treatment analysis of the subtrates after comminution showed that total available carbohydrate content was 95.7 ± 1.55 % that are used in ethanol production. Meanwhile, **(El-Tayeb et al. (2012)** reported total carbohydrate composition of 95.7 % *w/w* for bagasse.

Table 1

Biomass	**Total carbohydrate (% dry weight)**
Acid pretreated Orange peel before	75.8 ± 2.9 (w/w)
Acid Pretreatment orange Peel after	25.9 ± 0.2 % (w/w)
Toatal Dry weight of the sample	95.7 ± 1.55 %(w/w)

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Table 2

PRODUCTION OF ETHANOL

S.NO.	SAMPLE	STRAIN NAME	ETHANOL PRODUCTION ON FIFTH DAY (G/L)
1.	Orange Peel,	*Pseudomonas aeroginosa-**I***	8.80 ± 0.75 **(G/L)**
2.	Bamboo Leaves	*Bacillus clausii-**II***	9.03 ± 0.08 **(G/L)**
3.	Tapioca Shell	*Enterobactor cloaca-**III***	9.56 ± 0.45 **(G/L)**
4.	Orange Peeel as a sole substrate for the fermentation	*Bactetial Consortia strain **I+II+III***	0.91 ± 0.49 **(G/L)**

Conclusion

The present study indicates that ***Pseudomonas aeroginosa, Bacillus clausii, and Enterobactor cloaca*** is a novel bacterium were used for fuel ethanol production and that no earlier work has reported in bioethanol production from this bacterium. So, it may be very important in future studies in bioethanol-production technology. The utilization of orange peel for bioethanol production is a sustainable and eco-friendly approach for renewable biofuel production. Bioethanol can serve as an alternative source of energy and can overcome the problem of energy crisis in future. The optimum temperature for bioethanol production was 37C, from Orange peel as a substrate. Bioethanol is the best alternate source of fuel and considered as fuel of future. Exploration of low-cost substrate and use of an efficient microorganism will open new doors for the bioethanol-production technology. **(S. Tiwari., *et al,* 2015)**

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