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PRODUCTION OF BIOETHANOL USING *SACCHAROMYCES CEREVISIAE* ISOLATED FROM *IXORA COCCINEA* AND *HIBISCUS ROSA SINENSIS*

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ABSTRACT

Floral nectar which serves as an ideal habitat for yeasts and pigment producing bacteria due to its rich sugar content was explored. More than ten different species of bacteria, fungus and yeasts were observed in the nectar of *Ixora coccinea* and *Hibiscus rosa sinensis* flowers. The isolate showing abundant growth from the flower nectar was identified by morphological and molecular characterization as *Saccharomyces cerevisiae* was used for Bioethanol production. Sugarcane bagasse, an agricultural waste was used as a raw material for the production of bioethanol. The agricultural waste was grinded and liquified by alpha amylase and glucoamylase with the suitable preparation of buffer and the isolate was inoculated into the sample, fermentation process converted the present simple sugar in bagasse into ethanol and CO_2. Distillation and heating produced the final product, bioethanol. It was observed that its sugar concentration decreased (350mg-150mg) and the alcohol production increased (1.0ml-2.4ml) simultaneously. This technique is a very cost effective process to get an economically important product. Rather than just disposing or burning the sugarcane bagasse agricultural waste it can be used as biomass for bioethanol production.

KEYWORDS

Bioethanol, *Ixora coccinea, Hibiscus rosa sinensis*, *Saccharomyces cerevisiae* and Sugarcane bagasse.

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INTRODUCTON

Flower's nectar has long been thought as an ideal habitat for yeasts because of rich sugar contents and for many years yeasts were most believed to occur elsewhere. Despite the huge number of publications a nectar properties appearing in the last few decades, there is still one peculiar feature of floral nectar that remains largely unexplored to date from an ecological perspective, namely its role as a natural habitat for many kinds of microorganisms, and more specifically, yeast[1,2]. That yeasts are frequent inhabitants of floral nectar was already

familiar to microbiologists more than a century ago[3,4].

The fermentation of carbohydrate present in plant biomass, to ethanol is achieved by *Saccharomyces cerevisiae*. Yeast metabolizes carbohydrates and produces CO2 and ethanol as metabolic end product in an anaerobic condition. *Saccharomyces cerevisiae* is a species of yeasts (single-celled fungus microorganism). It is believed to have been originally isolated from the skin of grapes. It is one of the most intensively studies eukaryotic model organism in molecular and cell biology, much like *Escherichia coli* as the model bacterium. It is the microorganisms behind the most common type of fermentation. *Saccharomyces cerevisiae* cells are round to avoid, 5-10mm in diameter. It reproduces by budding.

Ethanol is also known as grain alcohol as it can be made from barley and wheat or from cellulose biomass such as wood, paper pulp or agricultural wastes. Human population has dramatically increased in the past decades, stretching the finite fossil fuels resources. Current bio fuels for bio ethanol and bio diesel production are based on sugar crops. Bio ethanol is bio fuel which is blended with gasoline in fixed proportion and used as alternative to fuel like diesel and petrol. Maximum of 20% bio ethanol can be blended with gasoline to be used as alternative fuel source in same carbonator engine[5]. Ethanol has been produced from varieties of substrates.

First, ethanol production from different wastes such as a molasses, sugar wheat pulp, waste from cassava starch production, food waste leachate, and waste newspaper has been reported. Ethanol production from wastes has to major advantages. On the other hand, it reduces or eliminates cost of waste disposal. Current bio fuels for bio ethanol and bio diesel production are based on sugar crops. The bio fuels produced from starch, sugar, animal fats and vegetable oils are referred as first generation bio fuel.

This present study aimed at isolation of microorganisms from *Hibiscus rosa sinensis* and *Ixora coccinea*. Ten isolated colonies with prominent growth were observed from both plants each. Isolated colonies were identified by morphological and biochemical characteristics. The dominant isolate from *Ixora coccinea* was identified by 18S r RNA sequencing protocol and identified as *Saccharomyces cerevisiae* and was used for bioethanol production from sugar cane bagasse. The main objectives of the experiment were:-

To isolate and identify microorganisms from *Hibiscus rosa sinensis* and *Ixora coccinea* crude nectar

Bioethanol production from sugarcane bagasse by the selected isolate *Saccharomyces cerevisiae*.

MATERIAL AND METHODS

Isolation and identification of microorganisms

Hibiscus rosa sinensis and *Ixora coccinea* flower were collected from plants in sterile glass jars at Sree Narayana Guru College, Coimbatore. The nectar was separated from individual flowers using calibrated micro capillaries. Crude nectar samples were spread plated onto Nutrient agar, Sabouraud dextrose agar and yeast extract peptone dextrose agar medium. The isolated colonies obtained from each plates were selected and subculture for the pure culture. Identification of microorganisms was done morphologically with simple, gram staining and various biochemical tests[6].

Identification of yeast was done by simple staining, LPCB, Carbohydrate fermentation test[6,7]. The confirmation of yeast was carried out by 18S rRNA sequencing.

Preparation of fermentation process

The sugarcane bagasse was obtained from Mundur, Palakkad district. It was cut into small pieces after washing with tap water and maintained it at 60°C for 3 days for drying. After drying it was grinded using grinding machine and transferred it into seal bag and store stored at room conditions. Buffer was prepared for the dilution of enzyme alpha- amylase and glucoamylase. There were two types of buffers prepared-phosphate buffer for alpha amylase and acetic acid with sodium acetate buffer for glucoamylase. The buffers were stored at room temperature. For liquefaction of sugarcane 0.2 microliters of enzyme alpha-amylase was added to the mixture of sugarcane powder and distilled water. The saccharification of sugarcane 0.2 microliters of glucoamylase buffer was added to the mixture. 10ml of *Saccharomyces cerevisiae* was added to mixture and fermented it for 48 hours.

After 48 hours the mixture was filtered using Whatsman No.1 filter paper to separate the ethanol from the mixture[8].

Confirmation of alcohol production

The confirmation of ethanol production was done by litmus test, iodoform test, ester test and also estimation of reducing sugar[6].

RESULTS AND DISCUSSION

The nectar of *Ixora coccinea* and *Hibiscus rosa sinnesis* (Figure No.1) was found to be an ideal space for observing many sugar loving microorganisms[9] showing similarity with previous works. Various different colonies were observed on Nutrient agar, SDA agar and YEPD agar plates (Figure No.2-4) (Table No.1 and Table No.2).

MICROSCOPIC OBSERVATION OF SELECTED ISOLATE

The isolated yeast organism (Figure No.4) appeared as unicellular, large spherical individual cells and taken crystal violet and appeared purple in colour. The cells taken up lactophenol cotton blue stain and appeared blue colour spherical to oval structure. Some of the cells also showed bud formation.

CARBOHYDRATE FERMENTATION TEST

Gas formation was observed in media inoculated with yeast. No gas formation was observed in control tube. Colour of the media also turned red to yellow. Glucose was fermented by the selected isolate. As it was confirmed by accumulation of gas in the Durham's tubes, and absence of gas formation and color changes in sucrose and mannose containing medium.

18S r RNA sequencing confirmed that the selected organism isolated from *Ixora coccinea* nectar on YEPD agar medium was *Saccharomyces cerevisiae* (Figure No.5). The selected isolate was used for further study.

Bioethanol production from *saccharomyces cerevisiae*

At odd days of incubation a sample (Figure No.6) was collected and estimated for the presence of remaining sugar and production of alcohol (Figure No.7). The sugar concentration decreased from initial of 350mg/ml to 150mg/ml about 50% reductions was observed. And at the same time alcohol production increased from 1.0ml to 2.4ml/10ml of which was relatively increase in volume. About 70% increase in alcohol content was recorded showing similarity to other works[10].

Confirmation of bioethanol production litmus test

After fermentation, it was qualitatively evaluated for the production of ethanol. Alcohol production was confirmed by change of litmus paper from Blue to red, indicating the presence of ethanol in the tested sample (Figure No.9).

Iodoform test

In iodoform test, a yellow colour precipitation was formed at the bottom of the test tube after the addition of iodine indicated the production of alcohol (Figure No.10).

Ester test

Fruity smell indicated presence of ethanol (Figure No.11).

ESTIMATION OF BIOETHANOL

Selected isolate from *Ixora coccinea* was identified as *Saccharomyces cerevisiae*[11] and was used for bioethanol production from sugarcane bagasse. Sugarcane bagasse is a major agricultural waste. This material does not have any nutritional value, easily available and cheap. It does not require any separate agricultural land, water supply, fertilizers and energy sources. Rather than just disposing or burning this waste it can be used as biomass for bioethanol production.

Sugarcane bagasse was collected. Phosphate buffer and acetic acid with sodium acetate buffer were prepared. α - amylase and glucoamylase were diluted with the prepared buffers. 10gm of sugarcane bagasse weighed, NaCl was added to adjust pH. α - amylase and glucoamylase were added and maintained at 50^{o}C. Then 10ml of *Saccharomyces cerevisiae* added to the sample and incubated for 15 days under closed environment at room temperature. The present study showed similarities with previous works on ethanol production from pineapple waste[12] when estimated for the presence of remaining sugar and production of alcohol. The sugar concentration was decreasing from initial of 350mg/ml to 150mg/ml about 50% reduction in sugar was observed. And at the same time alcohol production was increasing from 1.0ml

to 2.4ml/10ml which was relatively increasing in volume (Figure No.12). About 70% increase in alcohol content was recorded. Still one third of the sugar remained. It showed that enough potential was there to produce more amount of ethanol from remaining sugar.

Table No.1: Identification and characterization of isolates on nutrient agar

S.No	Flower	Colony Morphology	IMVIC				Oxidase	Catalase	Gram staining	Organism
			I	MR	VP	C				
1	*Ixora coccinea*	Round, smooth, convex, yellow orange colour colony	-	+	+	+	+	-	Positive cocci	*Staphylcoccus* sp
2	*Ixora coccinea*	white, circular	-	-	+	+	+	+	Negative rod	*Bacillus* sp
3	*Ixora coccinea*	Red colour colony	-	-	+	+	-	+	Positive rod	*Serratia* sp
4	*Hibiscus rosa sinensis*	pale, circular	-	+	+	-	-	+	Negative rod	*Acetobacter* sp
5	*Hibiscus rosa sinensis*	circular, smooth, yellow colour colony	-	+	-	-	+	+	Positive cocci	*Micrococcus* sp

+Positive growth, - No growth

Table No.2: Characterization of fungal isolates on SDA

S.No	Flower	Morphological Characteristics	Colony Morphology	Organism
1	*Ixora coccinea*	Cottony appearance initially whiteto yellow and then turning black with conidial production	Branched, Septatemycelium. Long, erectconidiophores. Conidiospore present.	*Aspergillus niger*
2	*Ixora coccinea*	Blue – green, powdery and paleyellow on the reverse	Hyphal conida andconidiophores, Greenspiked conidia. Conidia are produced in column chains.	*Aspergillus fumigates*
3	*Ixora coccinea*	Green dense raised growth	Septate, Long erect	*Aspergillus* sp
4	*Hibiscus rosa sinensis*	Rapid growing, flat, filamentous and velvety, wooly or cottony in texture. The colonies are initially white and become blue-green gray green or olive gray	Septate hyphae that giverise to branched conidiophore. Lookslike a paint brush.	*Pencillium* sp
5	*Hibiscu rosa sinensis*	Cottony appearance, Initially white to yellow and then turning black with conidial production	Branched, septatemycelium. Long erect conidiophore. conidiopore present	*Aspergillus niger*

Figure No.1: Collected *Hibiscus rosa sinensis* and *Ixora coccinea* flowers

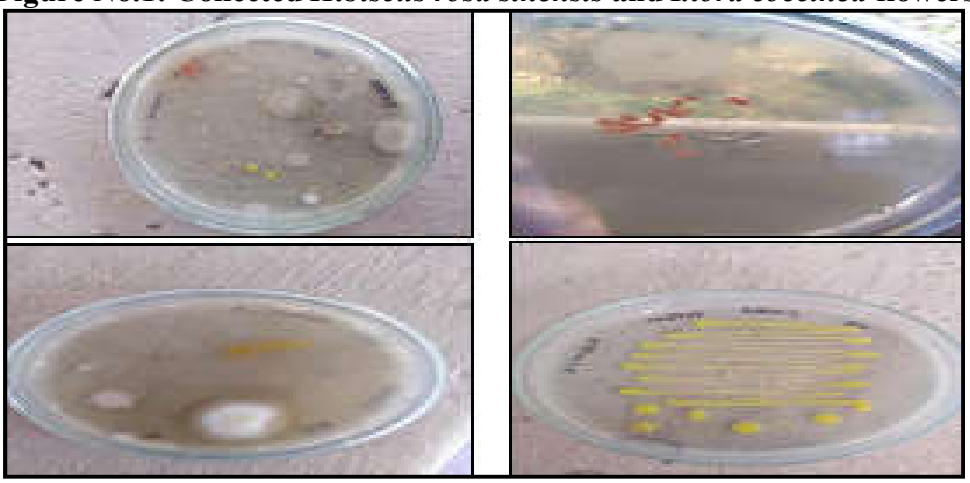

Figure No.2: Colonies isolated on nutrient agar plates

Figure No.3: Colonies isolated on SDA plates

Figure No.4: Yeast Colony isolated on YEPD plate

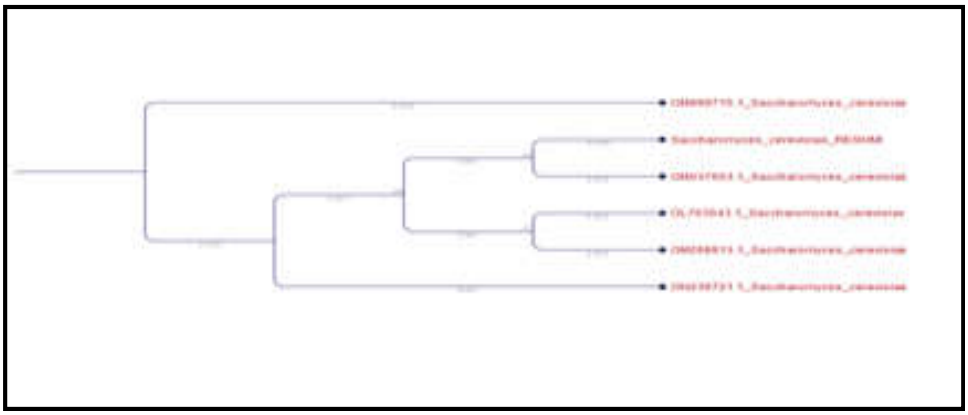

Figure No.5: 18s r RNA – Sequencing

Figure No.6: Collected sugarcane bagasse

Figure No.7: Extracted bioethanol

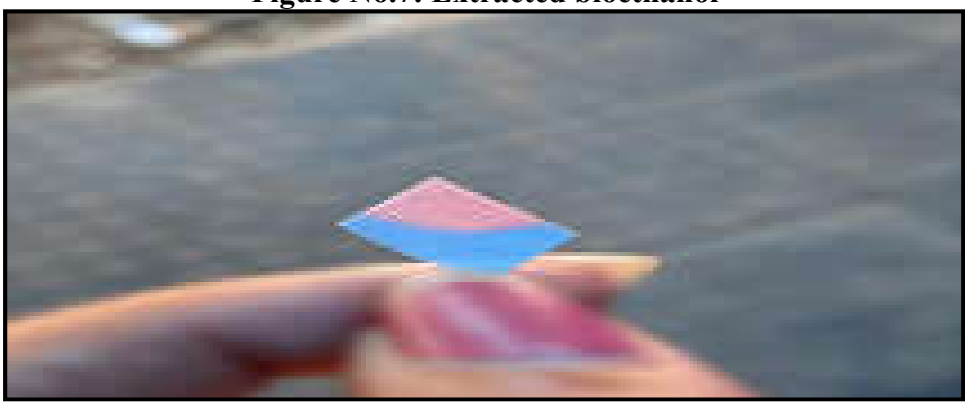

Figure No.9: Litmus test

Figure No.10: Iodoform test

Figure No.11: Ester test

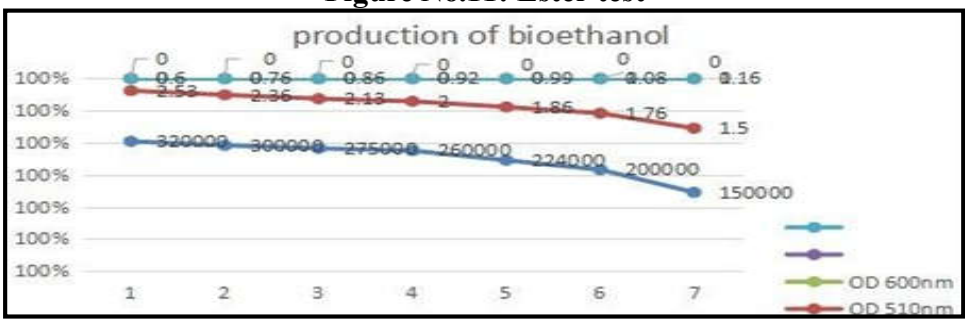

Figure No.12: Estimation of bioethanol

CONCLUSION

Ixora coccinea and *Hibiscus rosa sinensis* nectar contains variety of microorganisms. *Saccharomyces cerevisiae* used for bioethanol production was selected and isolated from *Ixora coccinea* nectar. *Ixora* sp. nectar can be easily collected currently in the world energy consumption is increasing with the ever increasing population. Bio fuels seem to be an efficient and sustainable energy resource. Sugarcane bagasse is an agricultural waste, which does not have any nutritional value, easily available and is cheap. It can be easily available and are cheap. So, bioethanol can be efficiently produced by *Saccharomyces cerevisiae* isolated from *Ixora coccinea* nectar can serve in a dual process by making a biofuel and cleaning an agricultural waste.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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