INDUCED MUTATIONAL STUDIES ON SACCHAROMYCES CEREVISIAE FOR BIOETHANOL PRODUCTION FROM FRUIT WASTE

Muralidhar Talkad[1], Chethan.C[2], Kavya.S[3], Qudsiya.S.S[4], Aamir Javed[5]

[1]*P.G. Department of Biotechnology, R&D Centre, Dayananda Sagar College of Biological Sciences, Kumaraswamy Layout, Bangalore-560078, India*
[2]*P.G. Department of Biotechnology, R&D Centre, Dayananda Sagar College of Biological Sciences, Kumaraswamy Layout, Bangalore-560078, India*
[3]*P.G. Department of Biotechnology, R&D Centre, Dayananda Sagar College of Biological Sciences, Kumaraswamy Layout, Bangalore-560078, India*
[4]*P.G. Department of Biotechnology, R&D Centre, Dayananda Sagar College of Biological Sciences, Kumaraswamy Layout, Bangalore-560078, India*
[5]*Jr.Embryologist.Base Fertility Medical Science Pvt, Ltd*

Abstract

Rising petroleum costs, trade imbalances and environmental concerns have stimulated efforts to an advance in the microbial production of fuels from lignocellulosic biomass. Ethanol, the most widely used renewable liquid transportation fuel, has only 70 % of the energy content of gasoline and its hygroscopicity makes it incompatible with existing fuel, storage and distribution infrastructure.

Saccharomyces cerevisiae is the only yeast that can rapidly grow under aerobic as well as in anaerobic conditions. This unique ability plays a major role in various industrial applications of S. cerevisiae, including beer fermentation, wine fermentation and large-scale production of biofuel ethanol. Here we focused on Induced Mutations for Yeast to attain better Bioethanol yield from Fruit waste.

We planned for other substrates such as the fruit waste example banana peel, chikko peel, mango peel, pine apple peel and other fruit waste. Ethanol estimated by chromic acid assay, Induction of mutation by EMS &UV. Ethanol estimated by Gas chromatography, Isolation of genomic DNA using mini prep filters.

The Results obtained from the Gas Chromatography for the Saccharomyces cerevisiae -EMS & UV Mutated, the Purity level for the Ethanol was found to be 73.5 % & 53.9 % respectively, with acid treatment it showed 53.9 %.The Genomic DNA bands of S. cerevisiae (control) and mutated (UV & EMS) has shown slight variation since, it could be responsible for the higher ethanol production, this method can be utilized for renewable source of energy & domestic wastes with sustainable technology.

Key words: *Bioethanol, Saccharomyces cerevisiae, mutation by EMS &UV, acid treatment, Gas Chromatography, Genomic DNA*

----------***----------

1. INTRODUCTION

Ethanol fuel production is a well-established commercial technology. But it is also a technology that has room to improve. That is why research and development efforts in ethanol fuel production are ongoing. The research areas relating to this technology that continue to be addressed include (1) feedstock; (2) starch hydrolysis and fermentation process design; (3) ethanol and by-product end and (4) site-specific integration of ethanol production with local agricultural economics. Feedstock is the most significant cost element in ethanol production. Questions of possible competition for prime agricultural land, and impacts of ethanol production on food supply and distribution are crucial to the social and economic success of this technology[1]**.** Research on integration of ethanol fuel production with agricultural economies could cover a broad range of topics, including feedstock economics and cultivation, plant and equipment design to fit specific local constraints, process fuel sources, impacts on employment and income distribution, and effects on national balance of payments[2]**.** Ethanol as a fuel is not optimal; it has low energy content (67% less than gasoline), requires energy to separate from water and is corrosive [3]**.** Therefore, the major goal for biofuel production is to develop an organism or community of organisms that utilizes all of the major components of lignocelluloses and produces a more gasoline-like fuel.

This project emphasis on the production and use of ethanol (ethyl alcohol) as a liquid fuel, the production of ethanol is a well-established technology; however, the use of ethanol as a liquid fuel is a complex subject[4]. By treating organic wastes such as the fruit waste and their peels such as the Mango peel, papaya peel, chikoo peel, pine apple peels and banana peels as a resource and applying a different method of waste management[5], organic wastes could contribute significantly to global energy needs. The ethanol distillation process has a waste product - spillage; a soup like waste stream that contains substantial organic content[6]. By digesting this material to optimum efficiency there are three resources recovered biogas (80% methane), biosolids (high in nutrients equivalent to high grade fertilizer) and recyclable water. Therefore if a waste treatment process could convert the waste into recoverable resources; such as methane; then the subsequent methane utilized back in the distillation / production process as a source of energy, this would essentially reduce the cost of producing ethanol in accordance with the true costs being considered [7]**.** Carbon now has a value per ton of emissions established in several markets around the world – these values can be attributed directly to energy use[8]**,** by utilizing waste treatment to recover resources, using those resources directly in the operation / process / production, valuing all externalities associated both foregone and utilized;[9]**.**

In 1908, ford unveiled his model T engine equipped with carburetor that could be adjust to use alcohol, gasoline and the mixture of both fuels[10]. Hence, the present study was framed to determine the effect of acid pretreatment, enzymatic saccharification by microbial enzymes and further ethanol fermentation of the obtained hydrolyzates from banana and mango fruit biomass by yeast. The proximate composition of fruit pulp and peels of Banana and Mango have also been studied in order to explore their potential application in bio-ethanol production**.**[11]**.** Some inhibitors present in the hydrolyzate, such as furans and phenolics, can be metabolized by yeast and converted to less inhibitory compounds. This capability is known as in situ detoxification capacity of yeast cells. The in situ detoxification capacity of yeast has been applied to develop a fed-batch process for cultivation of severely inhibiting hydrolyzates[12, 13].

A continuous mode of operation has been of interest to cultivate the hydrolyzates due to many advantages including higher conversion and faster fermentation rates, reduced product losses and higher volumetric ethanol productivity[14]. More importantly, the rate of inhibitors addition to the bioreactor can be controlled at the levels beyond the capacity of cells for in situ detoxification when toxic medium such as hydrolyzates are to be fermented.

S.cerevisiae displays the Crabtree effect: When it grows on glucose under aerobic conditions, the sugar is largely fermented to ethanol rather than respired. This effect is due in part to the repression at high glucose concentrations of genes encoding respiratory activities. Glucose repression is a complex regulatory system controlling numerous biochemical pathways. In this study, we investigated the role of GAL1 in ethanol production by comparing ethanol production and biomass of the wild-type strain and GAL1 mutants in glucose substrate [15]**.**Thus, nucleic acid based methods that identify species of *Saccharomyces* independent of morphology are now being developed to augment or replace morphological identification methods. Molecular methods were used for the identification of yeast from products like wine[16]. However, *S. cerevisiae* cannot transport and use xylose as a substrate, whereas the isomers of xylose (xylulose and ribulose) can be fermented [17]. Raw materials containing sugars or materials which can be transformed into sugars can be used as fermentation substrates.

2. MATERIALS AND METHODS

2.1 Microorganism Collection

The fungi *Saccharomyces cerevisiae*(NCIM NO.3570) used in this study were procured from National chemical laboratory, Pune. Cultures were maintained on MGYP medium.

2.2 Raw Materials (Substrate)

Fruit peels of Banana, Papaya, Chikoo, Mango and Pineapple were collected from a local fruit juice shop were chopped into small pieces and oven dried at 680c for 48hr. The dried peels were powdered in a mixer grinder and stored in bottle at room temperature.

Ethanol production estimated by chromic acid assay method in dried fruit peels without treatment followed by Estimation of reducing sugar by DNS method. Ethanol production estimated by chromic acid assay method from dried fruit peels with acid treatment

2.3 Induction of Mutation by EMS

Materials required:-*Saccharomyces cerevisiae*(NCIM NO.3570) Sterile water, MGYP plate, 0.1 M sodium phosphate buffer, pH 7.0, Ethyl methanesulfonate, 5% (w/v) sodium thiosulfate (autoclaved),13 × 100–mm culture tube, Vortex, 30^0c incubator with rotating platform

2.4 Grow and Mutagenize Cells

- Grow an overnight culture of the desired yeast strain in 5 ml MGYP medium at 30^0c.
- Determine the density of cell in the culture and record this number Adjust concentration to ~2×10^8 cells/ml if necessary. Transfer 1 ml of the culture to a sterile micro centrifuge tube.
- Pellet cells in a micro centrifuge for 5 to 10 sec at maximum speed, room temperature. Discard supernatant and resuspend in 1 ml sterile water. Repeat wash. After the second wash, resuspend cells in 1.5 ml sterile 0.1 M

sodium phosphate buffer pH 7.0.

- Add 0.7 ml cell suspension to 1 ml buffer in a 13 × 100–mm culture tube. Save remaining cells on ice for a control.
- Add 50 µl EMS to the cells and disperse by vortexing. Place on a rotating platform and incubate 1 hr at 30^0c. (EMS treatment should cause 40% of the cells to be killed).
- Transfer 0.2 ml of the treated cell suspension to a culture tube containing 8ml sterile 5% sodium thiosulfate, which will stop the mutagenesis by inactivation of EMS.
- If cells are to be stored before plating, pellet in a tabletop centrifuge 5 min at 3000×g at 4^0c, resuspend in an equal volume of sterile water and store at 4^0c.

2.5 Induction of Mutation by UV

Materials required:-UV germicidal light bulb (Sylvania G15T8; 254 nm wavelength) or Stratagene UV Cross linker, 23^0c incubator *Saccharomyces cerevisiae* (NCIM NO.3570), MGYP plate

- Grow an overnight culture of the desired yeast strain in 5 ml MGYP medium at 30^0c.
- Determine the density of cell in the culture and record this number Adjust concentration to ~2×10^8 cells/ml if necessary. Transfer 1 ml of the culture to a sterile micro centrifuge tube.
- Pellet cells in a micro centrifuge for 5 to 10 sec at maximum speed, room temperature. Discard supernatant and resuspend in 1 ml sterile water. Repeat wash. After the second wash, resuspend cells in 1ml of sterile water.

2.6 Plating

- Make serial dilutions of the culture in sterile water so that each plate has 200 to300 viable cells.
- Plate 0.1 and 0.2 ml of the diluted cells on separate sets of MGYP plates, using ten plates in each set. Incubate all plates for 3 to 4 days at room temperature.
- Irradiate all but two plates from each set with UV light using a dosage of 300 ergs/mm^2 (there should be 40% to 70% survival). The nonirradiated plates will serve as controls to determine the degree of killing by the UV light.

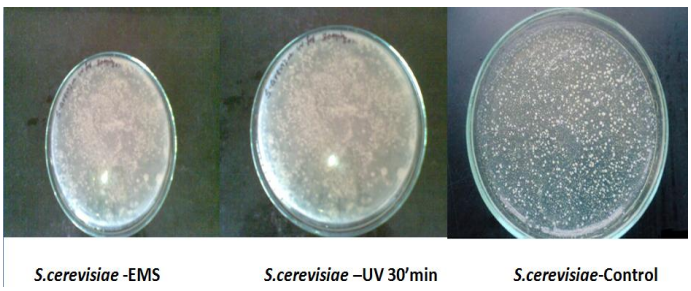

Fig: 1 Saccharomyces cerevisiae- mutagenized plates compared with control

2.7 Ethanol Production Estimation by Chromic Acid Assay Method from Dried Fruit Peels without Treatment with Mutated Culture

- 25 gm of mixed fruit peels powder (containing 5gm of each pineapple, mango, chikoo, papaya and banana) was weighed and dissolved in five 500ml conical flask each containing 250 ml distilled water and left for overnight at room temperature for sugar to release.
- The amount of free sugar released was determined by DNS method.
- The above content was autoclaved at 121^0c at 15lbs for 15 minute.
- The content was allowed to cool and 10% inoculums of 24hr old mutated(EMS+UV) culture *S.cerevisiae* were inoculated in five separate 500ml conical flask containing sterilize medium.
- The medium was incubated for 7days at 28^0c in rotary shaker for fermentation to occur.
- After 7 days of incubation the broth was filtered through muslin cloth and centrifuged at 5000rpm for 5 minute to remove cell and suspended particles.
- The supernatant was collected and distilled at 78^0c to get ethanol.

2.8 Ethanol Estimation by Gas Chromatography

- Ethanol production was analyzed by Gas chromatography using ECD detector. The mobile was carrier gas. Aliquots of 20µl were injected in to the capillary column. The flow rate was adjusted to 2bar, pressure 5lb/in2 and oven temperature oven temp--80C.

2.9 Genomic DNA Analysis by Agarose gel Electrophoresis Isolation of Genomic DNA using Mini Prep Filters

- Rock Amnion Bioscience KIT-DNA Isolation Take 5ml of 24hr old cultures of *Saccharomyces cerevisiae* (NCIM NO.3570)
- Centrifuge 2 ml each in the same eppendroff tube at 10000rpm for 1 min (twice).
- Discard the supernatant and keep the pellet.
- To pellet, do dry spin at 2000 rpm for 1 min.
- Drain out the remaining media by leaving at room temperature for 5 minute.
- To pellet add 800µl of the homogenizing buffer.
- Mix with pipette tips gently for uniform mixing.
- Keep the tube in water bath temp 55-65^0c.
- Now to the tube add 1ml of the lysis buffer and mix gently and keep it on water bath at 55-65^0c.
- Now centrifuge the tube at 10000rpm for the 1 min.
- Keep the Supernatant and discard the pellet (1800µl).
- Now add 600µl of the supernatant to the mini prep filters and centrifuge at 10000rpm for 1 min and through the

filtrate

- To the same mini prep filters and do the spin at 10000rpm for 1 min (thrice).
- Now to the column add 100µl of the wash buffer (twice).
- Now finally add 50µl of the 5mM of 50Ul of the Milli Q water to elude the final DNA.
- Observed DNA bands under trans- illuminator

3. RESULTS

The Gas Chromatography for the sample ***Saccharomyces cerevisiae* -EMS Mutated**, it was found that the Purity level for the Ethanol was found to be **73.5** % and the Retention Time [min] was found to be 0.783, Area [mV.s] 1184.2, Height [mV] 429 and Area [%] 40.00, the Gas Chromatography for the sample *Saccharomyces cerevisiae* – with acid treatment it was found that the Purity level for the Ethanol was found to be **53.9 %** and the Retention Time [min] was found to be 0.857, Area [mV.s] 2000.19, Height [mV] 700.9 and Area [%] 76.90, the Gas Chromatography for the sample ***Saccharomyces cerevisiae* –Without Acid treatment** it was found that the Purity level for the Ethanol was found to be **29.4 %** and the Retention Time [min] was found to be 0.883, Area [mV.s] 3133.8, Height [mV] 1093.3 and Area [%] 92.6, the Gas Chromatography for the sample ***Saccharomyces cerevisiae* – Mutated UV** it was found that the Purity level for the Ethanol was found to be **53.9 %** and the Retention Time [min] was found to be1.593, Area [mV.s] 2009.3, Height [mV] 732.3 and Area [%] 98.9.

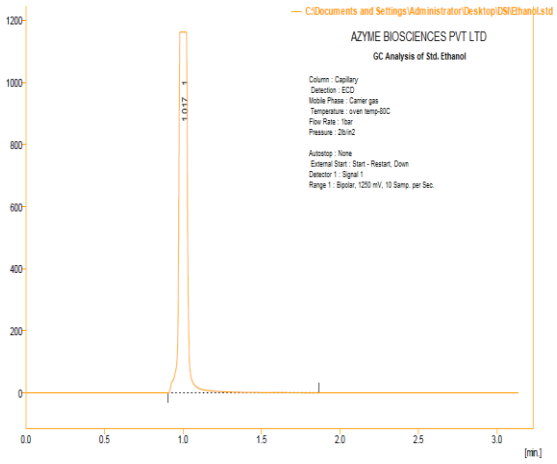

Fig: 2 the analytical reagent absolute ethanol was used as a standard

.

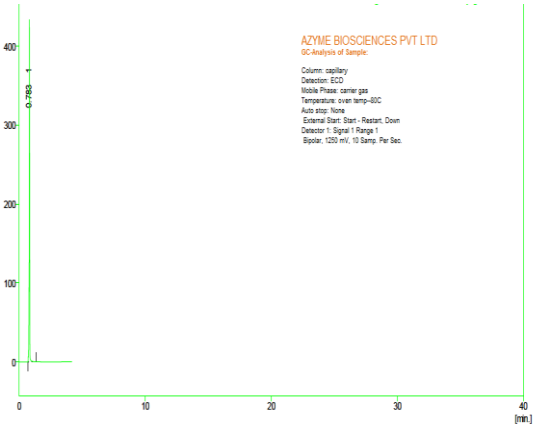

Fig: 3 Saccharomyces cerevisiae -EMS Mutated

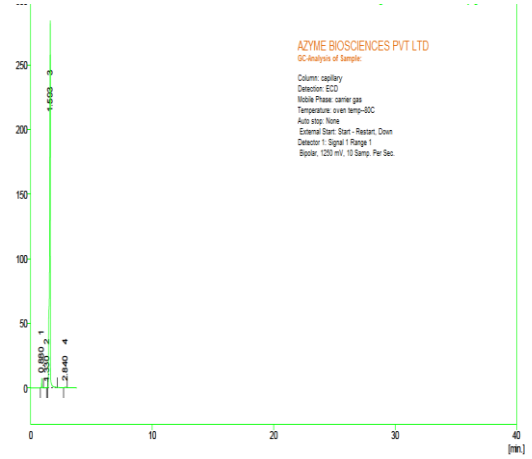

Fig: 4 Saccharomyces cerevisiae – Mutated UV

Note the Fig: 1. for *Saccharomyces cerevisiae*- was subjected for EMS & UV mutagenized plates compared with control. **GC analysis figures:** The analytical reagent absolute ethanol was purchased from Changshu YangYuan China and was used as a standard (fig 2)**.** Fig: 3 are *Saccharomyces cerevisiae* -EMS Mutated and Fig: 4 were *Saccharomyces cerevisiae* – Mutated UV

4. DISCUSSION

The amount of Reducing Sugar was found to be 500 mg /ml and 125g/250ml of sample without acid treatment. The amount of reducing sugar was found to be 750mg/ml and 187.5g/250ml of sample with acid treatment.Hence from the above result it can be concluded that the amount of reducing sugar is more in acid treated sample compared to without acid treated sample ,which means that the effect of the acid treatment on fruit peel i.e,lignocellousic materials breaks down the complex polysaccharides (Cellulose, Hemicellulose & Lignin) to simple sugar(Glucose & Fructose) , due to which the amount of sugar present in the acid treated sample is found to be more compared to the none acid treated sample ,which can be finally estimated by the DNS Method .

Observation from Chromic Acid Assay revealed the percentage of ethanol produced by *S.cerevisiae* for non acid treated 12 % and,the percentage of ethanol produced by *S.cerevisiae* for acid treated sapmple was found to be 12 % respectively. Hence from the above result it shows that the percentage of ethanol produced from acid treated sample is more compared to non acid treated sample, because of more amount of simple sugar present in the acid treated sample .

5. CONCLUSIONS

In this present study, efforts were made to identify the fruit wastes as potential raw material for bio ethanol production and the results showed that mixed ripened fruit biomass of banana and mango can yield a good percentage of ethanol. The main objective of our research was to induce mutation on the organism.

The mutation processes in carried out by EMS and UV. Finally the amount of ethanol produced by Saccharomyces cerevisiae on EMS treatment is 73.50 %, the amount of sugar present in non acid treated sample is 125gm/250ml and amount of sugar present in acid treated sample is 187.5gm/250ml.

ACKNOWLEDGEMENTS

The authors are extremely grateful to Dr. Premchandra Sagar, Vice Chairman, Dayananda Sagar Institutions and Dr. Krishna Gowda, Director, Dayananda Sagar College of Biological Sciences, Bangalore-560078, India, for their immense guidance and support for this project

REFERENCES

[1] Joshi, S.S., Dhopeshwarkar, R., Jadhav, U., Jadhav, R.,D'souza, L.,& Dixit, J. Continuous ethanol production by fermentation of waste banana peels using flocculating yeast. I J CHEM T. **2001.** 8(3): 153 156.

[2] Akin-Osanaiye, B.C., Nzelibe, H.C., &Agbaji, A.S. Production of ethanol from (pawpaw) agro waste: effect of saccharification and different treatments on ethanol yield. African Journal of Biotechnology. **2005.**. 4: 57 59.

[3] Somerville C. Biofuels. Curr Biol. **2007**. l 17(4):9. .

[4] Krishna S H and Chowdary G V, Optimization of simultaneous saccharification and fermentation for the production of ethanol from lignocellulosic biomass. Journal of Agricultural and Food Chemistry, **2000** 48:1971– 1976,.

[5] Hammond, J.B., Egg, R., Diggins, D.,&Coble, CG. .Alcohol from bananas. **1996** 56: 125 130.

[6] Gould J M, Alkaline peroxide delignification of agricultural residues to enhance enzymatic saccharifcation, Biotechnology and Bioengineering, **1984** 26:46–52..

[7] Yu Z and Zhang H,Ethanol fermentation of acid-hydrolyzed cellulosic pryolysate with S. cerevisiae, Bioresource Technology, **2004** 93:199–204.).

[8] Sheoran A, Yadav B S, Nigam P and Singh D, Continuous ethanol production from sugarcane molasses using a column reactor of immobilized Saccharomyces cerevisiae HAU-1, Journal of Basic Microbiology **(1998).**,38: 123–128,

[9] Martín C, Galbe M, Wahlbom C F, Hahn- Hägerdal B and Jönsson L J, Ethanol production from enzymatic hydrolysates of sugarcane bagasse using recombinant xylose-utilizing accharomyces cerevisiae, Enzyme and Microbial Technology, **2002** 31: 274– 282, .

[10] Solomon, B. D., et al. Grain and cellulosic ethanol: History, economics, and energy policy. Biomass & Bioenergy **2007** 31: 416-425.

[11] R.Arumugam, M.manikandan, Fermentation of pretreated hydrozylates of banana and mango fruit waste s for ethanol production/Society of Applied Sciences/Asian .J. Exp, Biol. Sci. vol 2(2) **2011**

[12] Nilsson, A., et al. Use of dynamic step response for control of fed-batch conversion of lignocellulosic hydrolyzates to ethanol. Journal of Biotechnology **2001** 89: 41-53.

[13] Taherzadeh, M. J., et al., Conversion of dilute-acid hydrolyzates of spruce and birch to ethanol by fed-batch fermentation. Bioresource Technology **1999b** 69: 59-66..

[14] Verbelen, P., et al. Immobilized yeast cell systems for continuous fermentation applications. Biotechnology Letters **2006** 28: 1515-1525.

[15] Rajkumar V. Raikar/ Enhanced Production of Ethanol from Grape Waste/ International Journal of Environmental Science/ **2012** Vol 3 No 2 / Issn- 0976-4402.

[16] Lopez, V., M.T. Fernandez-Espinar, E. Barrio, D. Ramon and A.Querol, A new PCR-based method for monitoring inoculated wine fermentations. Int. J. Food Microbiol., **2003.**81: 63-71.

[17] Jeffries, T. W., Engineering yeasts for xylose metabolism. Current Opinion in Biotechnology **2006.**17: 320-326.

BIOGRAPHIES

Dr. Muralidhar. T.S. Professor & Head. Post Graduate, Dept of Biotechnology. Dayananda Sagar Institutions, A doctoral degree holder in Reproductive behavior and Pathophysiology. Nearly 24 years of Research and Teaching experience in Toxicology, Phyto-Pharmacology, Biotechnology, Histopathology and Herbal Products. Has contributed 27 Research Articles and merited publication in International and National journals

Mr.Chethan Chowdary. P.G. Student of Biotechnology, Actively involved in academic and Research activities, he attended many seminars and presented papers, for his dissertation in MSc – he is working on microbial degradation of plastic and domestic waste with relative microbial gene expression and mutation induction studies.

Ms. Kavya Sudarshan.P.G. Student of Biotechnology, Actively involved in academic and Research activities, she attended many seminars and presented papers, for her dissertation in MSc –She is working on microbial degradation of plastic and domestic waste with relative microbes, followed by mutation induction studies.

Ms. Qudsiya Sayed Siraj. P.G. Student of Biotechnology, Actively involved in academic and Research activities, she attended many seminars and presented papers, for her dissertation in MSc –she is working on microbial Degradation of plastic and domestic waste with relative microbes, i.e., Pseudomonas followed by UV & EMS induced mutation studies.

Mr. Aamir Javed, Working as a Jr. Embryologist, Base Fertility Medical Science Pvt, Ltd. He has completed his Masters in Biotechnology, and a specialization in Synthetic Biology. He is presently using assisted reproductive technologies (ART) to help with infertility. An active researcher, who has 12 International Publications, 8 Copyrighted to his credit, one of his paper got published in project Phoenix Portugal.