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Ethanol tolerant *Zymomonas mobilis* for bioethanol production from vegetable wastes

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Abstract
The pollution load on environment is unmanageable and dangerous to human being. The vegetable wastes dumped in open areas are alarming as they cause health problems in nearby residential areas. Here, the main objective is to develop easier techniques and using cheaper source for the production of ethanol, which will reduce the wastes accumulation. This study employs the powdered vegetable wastes as the raw material for the production of ethanol. The produced Bio-ethanol content from the hydrolysate of powdered vegetable wastes was analyzed by FTIR spectroscopy. In order to reduce the complexity of vegetable wastes *Zymomonas mobilis* was added to the wastes for decomposition. The results indicate that the ethanol production rate through fermentation of vegetable waste is optimal at pH 4, temperature 30°C, specific activity 7 %. The results of this study show that vegetable wastes contains enormous amount of fermentable sugar and hence it can be converted into ethanol, which is used as an alternative energy source.

Keywords: *Zymomonas mobilis*, Bioethanol, Fermentation, vegetable waste

1. Introduction
Ethanol, also called as ethyl alcohol, is a colourless liquid fuel. The fermentation of sugar into ethanol is one of the earliest organic reactions employed by humanity. In modern times, ethanol intended for industrial use is produced either through the hydration of ethylene, or biologically by fermenting sugar or starch with yeast. Ethanol can also be obtained from fermentation of cellulose. Hence, second generation ethanol is derived from lignocellulosic materials (Taherzadeh, and Karimi, 2007) [1]

Bioethanol has emerged as the most suitable renewable alternatives to fossil fuel as their quality constituents match diesel and petrol (Tiwari *et al*., 2013) [2]. CO_2 emissions from road traffic worldwide will increase by 92% between 1990 and 2020. (Nejadkoorki *et al.*, 2008) [3]. It can be produced either from petrochemical feed stocks by the acid-catalyzed hydration of ethylene, or from biomass feed stocks through fermentation. On a global scale, synthetic ethanol accounts for about 3-4% of total production while the rest is produced from fermentation of biomass mainly sugar crops, e.g. cane and beet, and of grains (mainly corn).

Blending ethanol with gasoline can also oxygenate the fuel mixture so it burns more completely and reduces pollution emission. Ethanol fuel is widely sold in the United States. The most common blend is 10% ethanol and 90% petrol (E10) and vehicle engines require no modification to run on E10 and vehicle warranties are unaffected also (Lin, 2006) [6]

The most efficient microorganisms for converting glucose into ethanol are industrial yeast strains of *Saccharomyces cerevisiae* and bacterial strains of *Zymomonas mobilis*. Alcoholic fermentation is the main activity of yeasts, while *Saccharomyces cerevisiae* is the major species used in wine making (Alder, 1981) [6]. It utilizes sucrose, glucose, fructose, maltose and maltotriose as carbon sources to produce alcohol under anaerobic conditions. *Zymomonas mobilis* uptake glucose and ethanol production, higher ethanol yields and ethanol tolerance (Lee *et al*., 1979 and Rogers *et al.*, 1980) [5, 4].

India stands second in the production of fruits and vegetables in the world and it nearly generates 350 million tones of waste from the vegetables, fruits and other organic materials (Pappu *et al*., 2007) [15]. About 18 per cent of the fruit and vegetables production worth Rs. 44,000 core is going waste annually in India (2012 data). The level of fruits and vegetable processing is dismally 4%. While only 20% of fruits and vegetables are exported, most of our production caters to the defense, institutional sectors and household consumption. Also the fruit and vegetable processing industry in India is highly decentralized. A large number of units are in the cottage/home scale and small scale sector, having small capacities up to 250 tones/annum. (Shalini Gaur Rudra *et al*., 2015) [14]

Pretreatment is an important tool for practical cellulose conversion processes and is required to alter the biomass size and structure as well as its microscopic chemical composition and structure to enhance digestibility and enzymatic hydrolysis of the fibers to produce fermentable sugars (Mosier *et al*., 2005. Agbogbo *et al*., 2007) [10, 11]. An effective pretreatment must meet the following requirements: (Sukumaran *et al*., 2009) [9] improve the formation of sugars or the ability to subsequently form sugars by enzyme hydrolysis; (Mosier *et al.*, 2005) [10] avoid the degradation or loss of carbohydrate; (Agbogbo *et al.*, 2007) [11] avoid the formation of by-products inhibitory to subsequent hydrolysis and fermentation processes; (Prasad *et al*., 2007) [12].

2. Materials and methods
3. Collection and Preparation of Vegetable waste
Vegetable waste were collected from local market in Trichy,

air-dried then chipped, ground and sieved to size (0.5 – 1 cm). Ground materials were then stored in plastic bags at room temperature until analysis and treatment. Vegetable waste was initially analyzed for determination of hemi-cellulose, cellulose, and lignin contents.

4. Determination of pH and Temperature

Estimation of optimum temperature and pH were done at four different pH levels (pH 2,4, 6, and 8). Determination of growth was performed on spectrophotometer at 600 nm. The initial optical density of each tube was recorded on spectrophotometer at 600 nm against the medium as blank. All cultures were incubated at 25°C, 30°C, 35°C, 40°C and 45°C for observing thermo tolerance of bacterial strain. The increase in optical density in a tube was recorded as evidence of growth. Without it, growth on yeast extract medium agar media at 25°C, 30°C, 35°C, 40°C and 45°C was also observed to ensure thermo tolerance of the strain.

5. Preparation and pretreatment of substrate

Acid pretreatment of substrates was carried out by using dilute sulphuric acid. Ten grams each of cellulosic wastes was soaked in 100 ml of different concentration of H_2SO_4 (2-8%) separately and incubated at room temperature for 10 hr with an agitation of 150 rpm and autoclaving at 121^{o}C for 30 min. The residues were collected and washed extensively with tap water until neutral pH was reached, filtered and dried at 65^{0}C for two days.

6. Estimation of cellulose content in substrate

Three milliliter of acetic/nitric reagent was added to 1g of substrate and mixed well. The tube was placed in water bath for 30mins. The contents were centrifuged for 15-20 mins. To the supernatant 10 ml of 67% sulphuric acid was added and allowed to stand for one hour. The solution is diluted to 100ml. From the diluted solution, 1ml was taken and 10 ml of anthrone reagent was added. Test tubes were kept in water bath for 10 mins and measured at 630nm. After estimation of cellulose efficiently pretreated substrates containing higher amount of cellulose is observed under FTIR spectroscopy.

7. Fermentation

The commercial media was prepared by supplementing yeast extract,1 g (Glucose, ammonium sulphate, potassium dihytragen phosphate, magnesium chloride) each 1 ml organism added the 100 ml conical flask. The substrate was weighed 2 g and added into the media individually. The media solution was autoclaved and it is utilized. It was individually inoculated with isolate ethanol tolerant bacteria strain. The mouth of flasks were sealed and kept in a shaker incubator and allowed to incubate for 5 days at 30^0 C with a speed of 100 rpm. Samples were skeptically withdrawn at every 24 hours for estimation of bioethanol production.

8. Estimation of ethanol by potassium di chromate

Different concentration of alcohol in double distilled water was prepared starting with 1%, added 1 ml of the alcoholic solution to 24 ml of distilled water available in conical flask. Twenty five milliliter of sample to distillation flask was poured and distilled the contents. Ten milliliter of distillate was collected in a beaker containing 25 ml of 3.4 % chromic acid. The volume was made to 50 ml using double distilled water and mixed thoroughly. Contents was boiled up to 80^{0}C for 15 minutes. read the absorbency at 580 nm.

9. Estimation of reducing sugars

The reducing substance (sugar) obtained was determined by DNS method (Miller *et al.*, 1959) [17].

10. Results

11. Substrate Pretreatment H_2SO_4 Pretreatment

In acid-treatment H_2 SO_4 treated vegetable waste showed variations in the cellulose content at different concentrations. Among the treated substrates 6% pretreated vegetable.

12. Detection of pH and Temperature

Isolated bacterial strains were incubated for 48 hrs at 25^{0}C, 35^{0}C, 40^{0}C and 45^{0}C. Yeast strains were able to grow at 25^{0}C to 40^{0}C but strains were able to grow at 45^{0}C. Higher growth was found at 30^{0}C. The optimum pH for maximum amount of pH for the growth of bacterial strains was about at pH 4. (Palanivelu, 2004). (Fig:1 & 2)

13. Substrate collection and pretreatment

Vegetable waste collected from Trichy. The collected substrates were pretreated with acid pretreatment.

14. Estimation of cellulose content in substrate

Cellulose content in substrates was estimated using nictric/ acetic reagent. Cellulose content was higher of 108 mg in 6% H_2 SO_4. Vegetable waste treatment 6% H_2 SO_4 treatment of 92 mg cellulose.

15. FTIR analysis

The FTIR spectroscopy is an appropriate technique to establish the variations introduced by the different treatment of on the chemical structures of cellulose. All spectra were dominated by the peaks at 3501 cm^{-1} and 1484 cm^{-1} that correspond to the stretching vibrating of O-H in cellulose and C-O in hemicelluloses and cellulose, respectively. The peak at 1627cm^{-1} in all samples was indicative of the C=O bonds of hemicelluloses. Peaks observed at 1627cm^{-1} also showed the presence of hemicelluloses. Lignin peak values range from 1542 and 1484 cm^{-1} was absent in pure cellulose powder and pretreated cellulosic wastes. Pure cellulose powder was considered as the control for untreated and pretreated cellulosic wastes. (Fig 3 & 4)

16. Estimation of ethanol by potassium di chromate

The amount of ethanol produced was estimated by the potassium dichromate method with absolute ethanol as standard. The standard graph was prepared by dissolving ethanol in distilled water to get 1 to 15 % of ethanol concentration. The media was estimated at every 24 hours, during the process of fermentation, ethanol production increased with increase in time from 1 day to 5 days of incubation. Ethanol production was vegetable waste used as substrates of 7 %.

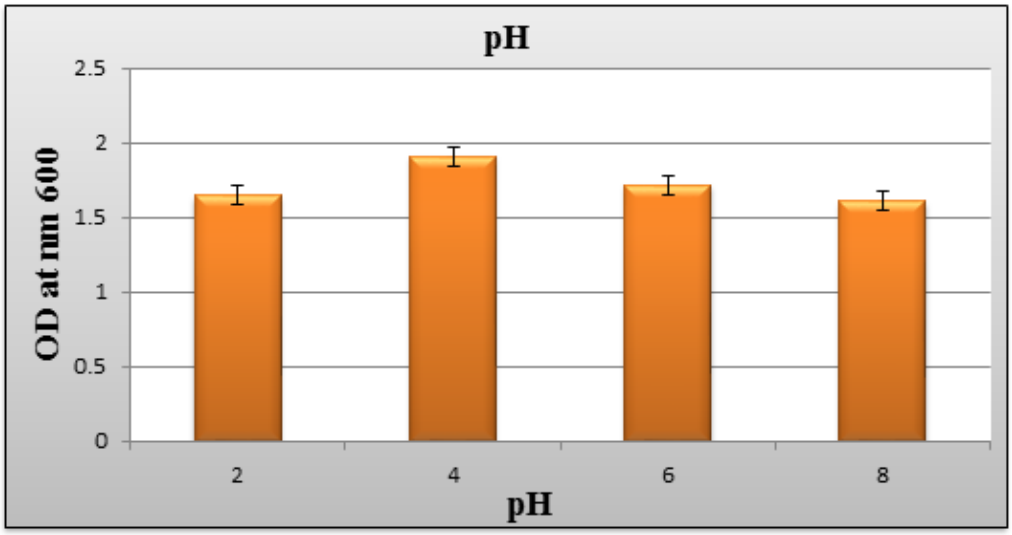

Fig 1: Optimization of pH

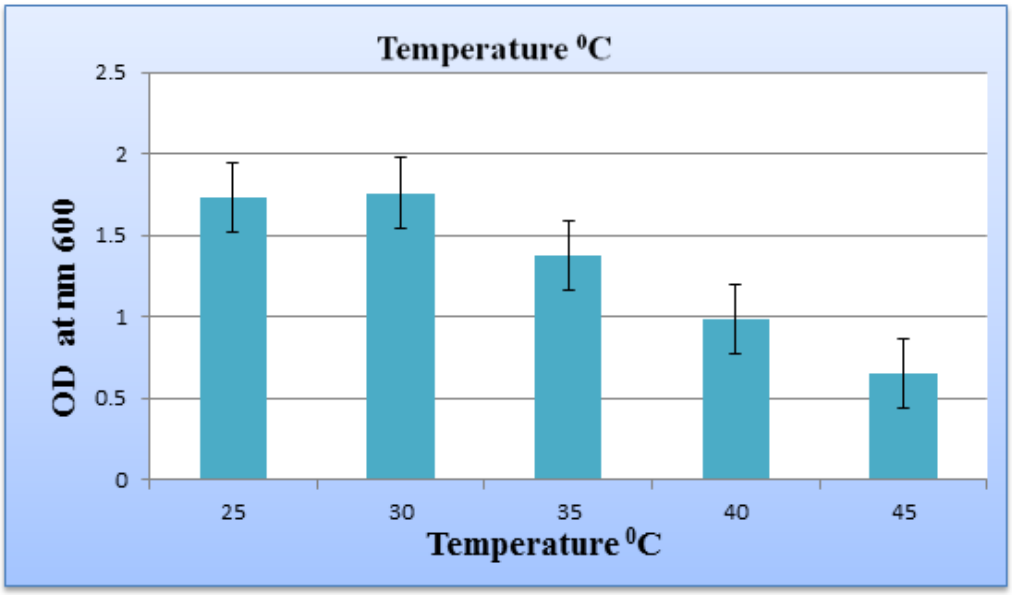

Fig 2: Optimization of Temperature;

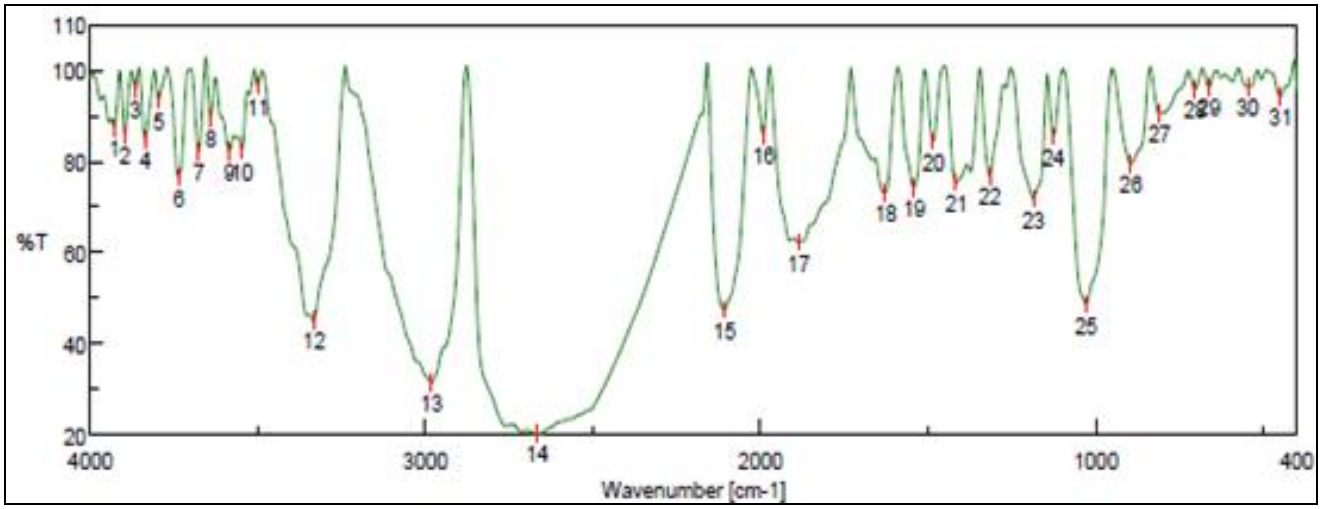

Fig 3: FTIR analysis of pretreated substrate 1. FTIR analysis of standard cellulose

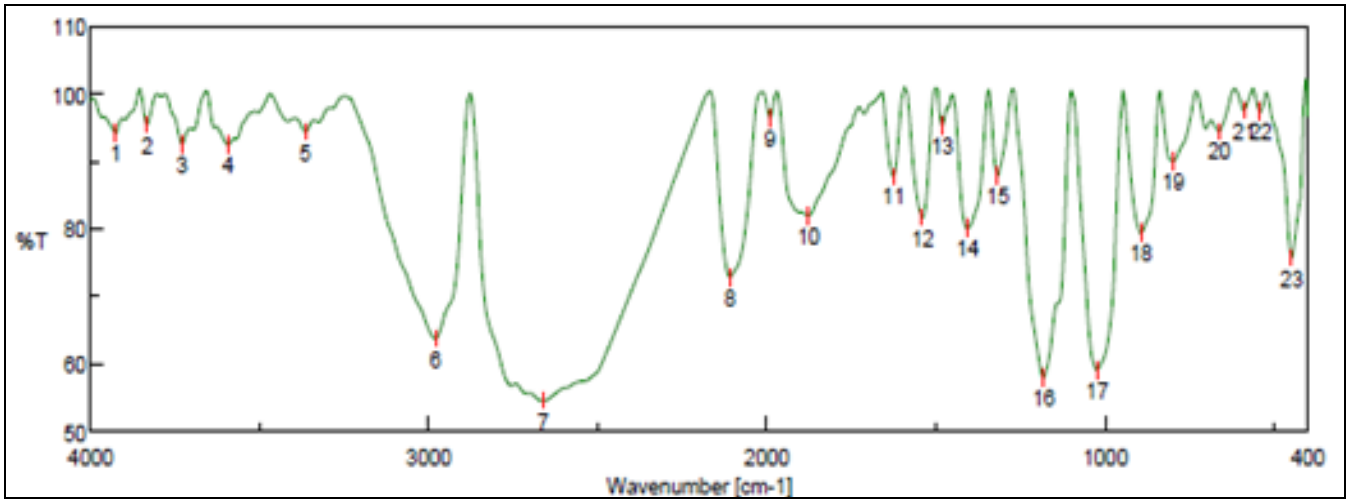

Fig 4: FTIR Analysis for 6% H_2SO_4 Vegetable waste

17. Discussion

Based on the colony characteristics (white and creamy texture) avoid microscope shape, the presence of ascospore, and budding pattern (multipolar), the selected isolate (grapes) were found to belong *Zymomonas mobilis* type unicellular ascomycete according to (Lodder, 1971) [24].

The isolates were tested for fermentation of carbohydrates and grape juice strain was capable to ferment five sugars out of the seven sugars tested. Glucose,Sucrose, Fructose, Lactose, and Trehalose were successfully fermented by this strain but it can't ferment Xylose and maltose. The Grapes failed to ferment Maltose and Xylose, but utilized five other carbohydrates. which proved the identity both of the microorganisms are *Zymomonas mobilis***.**

The vegetable waste substrate for bioethanol production and was powdered in a ball mill. These residues were done acid and alkali pretreatment method for the breakdown of lignin**.** The commercial and production and the yeast strain was individually inoculated for the ethanol production. The NaOH treatment was the best method for improving the use of vegetable waste. The effective pretreatment for individual substrates varies as it depends on the nature and condition of pretreatment. For vegetable waste, 6% NaOH released high percentage of cellulose respectively.

The differences were the results from the intra- and intermolecular degradation of hemicellulose during NaOH pretreatment. The intramolecular degradation of hemicellulose was represented by the decreased contents of functional groups and the disappearance of some bonds after NaOH pretreatment.

A strong broadband at 3422 cm^{-1} was found, which is attributed to the hydroxyl groups in the hemicellulose from both the untreated and NaOH-treated rice straws. The intensity of the peak decreased after NaOH and NaOH treatment, because of the disruption and breakage of hydrogen bonds. In the carbonyl stretching region, the absorption at 1631-1641 cm^{-1} is principally associated with absorbed water. The linear and branched (1 - 4)-*β*-xylans, such as glucuronoxylan and arabinoxylans, showed the main peak maximum at about 1044 cm^{-1}. A small peak at 1512 cm^{-1} was found in two spectra, which is mainly due to the presence of a small amount of associated lignin in hemicelluloses. (Sun *et al.*, 2005) [22].

In the present study, the optimum pH was observed at 4 pH, Temperature 30^0C for *Zymomonas mobilis* and growth for maximum bioethanol production. Then the media was aseptically withdrawn at every 24 hours and the amount of ethanol produced was calculated by the potassium dichromate method. After 24 days of incubation, pretreated substrate media and non treated media yielded. Ethanol production was vegetable waste used as substrates of 7 %.

18. Conclusion

Bioethanol production from vegetable waste and optimization of different factors in the fermentation process were investigated. The result of this study has shown that how the deferent parameters affect the production of bioethanol. From this study, it is clear that the maximum yield of ethanol was obtained at Temperature 30^0C and pH 4, 7%. From this study we conclude that the process is cheaper and does not produce any toxic residues. This bioethanol production process can be used for small and large scale production because vegetable waste can be obtained from rice industries continuously.

19. Acknowledgement

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