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Production of Bioethanol from Hydrolysate of Soaking in Aqueous Ammonia Pretreated Cassava Stem using Random Mutation of Zymomonas Mobilis

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Abstract: *Cassava is one of the major food crops in tropical countries after maize and rice. Cassava processing industry produces enormous amount of waste in the form of leaves, petioles, stem, rhizome, peel and bagasse. In this study, hydrolysate prepared from soaking in aqueous ammonia pretreated cassava stem is used as a substrate for bioethanol production. The residue obtained after pretreatment is subjected to microbial fermentation. The main aim of the work is to investigate ethanol production by subjecting Zymomonas mobilis to random UV mutagenesis technique by exposing to UV light at60 cm for 60 min. During the fermentation, ethanol concentration was compared between native and mutated Z. mobilis by varying the process variables such as pH (3 - 11), substrate concentration (2 -10 g/L), inoculum size (2 - 10% (v/v)), temperature (27– 47 °C), agitation speed (0 - 100 rpm), and time (12 – 120 h)which were optimised for native and modified strains to produce maximum ethanol production. A maximum ethanol concentration of 2.95and 2.00g/L were achieved for modified and native strains. Hence, mutated strain random UV mutagenesis is proved to be a potential technique for ethanol fermentation using Z. mobilis.*

Keywords: *Cassava stem, Random mutagenesis, Zymomonasmobilis, Bioethanol*

1. INTRODUCTION

Bioethanol is an alternate liquid transportation biofuel to petrol as it reduces the reliance on fossil fuels and enhances biomass-based economy [1]. Bioethanol is called first-, second- and third-generation fuel based on the feedstocks from which it is produced. Sugary and starchy feedstocks are used to produce first generation bioethanol. Cellulosic and hemicellulosic feedstocks are used to produce second generation bioethanol. Third generation bioethanol is produced from algal feedstocks [2]. Starchy, cellulosic and hemicellulosic feedstocks undergo hydrolysis and fermentation to produce bioethanol. Sugary feedstocks are fermented using ethanologenic microorganisms for bioethanol production [3].

Cassava is a major food crop in tropical countries after maize and rice [4]. Cassava processing industries generate huge quantities of waste in the form of leaves, petioles, stem, rhizome, peel and bagasse. Cassava stem is the highest proportion of waste produced from the crops [5]. Cassava stem, a lignocellulosic biomass of angiosperm,is rich in hemicellulose and cellulose, which makes them suitable for bioethanol production[6]. Hemicellulose and cellulose are hydrolysed through pretreatment and enzymatic hydrolysis, respectively, and fermented to produce bioethanol [7].

S. cerevisiae and *Zymomonas mobilis*are the most widely used ethanologenic microorganisms. Eventhough *S. cerevisiae* has been used in large-scale ethanol production;*Z. mobilis*need not possess controlled addition of oxygen when fermentation occurs and has been proved to have lesser biomass production, more sugar consumption, tolerance for ethanol and productivity than *S. cerevisiae*in the lab scale. Also, *Z. mobilis*employs the Entner-Doudoroff (ED) pathway to degrades sugars to pyruvate. Ethanol is further produced from this pyruvate along with carbon di oxide by fermentation [8]. The enzymes required for glycolysis and the pentose-phosphate pathway are absent in *Z. mobilis*. Because the ED route produces just one ATP molecule, *Z. mobilis* grows at a slower pace when

ethanol productivity increases. Because an increase in biomass is regarded a waste of resources in the context of biofuel production, this is one of the advantages of employing *Z. mobilis* to synthesise ethanol [9].

Irrespective of many lucrative advantages, there are couple of factors that inhibit the commercial production of ethanol through Z.mobilis. The primary being its limited substrate range to glucose, fructose and sucrose. Fermentation of pentose sugars such as xylose and arabinose that are major components of lignocellulosic hydrolysates cannot be accomplished by Wild-type *Z. mobilis*. Also,*Z. mobilis*couldn't endure toxic inhibitors like acetic acid and phenolic compounds that are present inlignocellulosic hydrolysatesunlike *E. coli* and yeast.[10]. Many efforts have been put forth togenetically modify*Z. mobilis* to prevail over its inherent deficiencies. However, the productivity and the yield doesn't match the industrial needs when fermentation of mixed sugars occur in the presence of inhibitors.This largely affects application of *Z.mobilis* for industrial purposes. An all-encompassing alteration process was used to enhance xylose fermentation in *Z. mobilis*. Similar to eukaryotic sterols, the plasma membrane of *Z.mobilis* consists of hopanoids, pentacyclic compounds. This lets it to have an exceptional tolerance to ethanol of around 13% in its environment, [11].

Some of the mutagenic methods include protoplast fusion, gene transformation, random mutagenesis, etc. Random mutagenesis can be brought about by physical, chemical or biological mutagens. Mutations induced by physical mutagens such as UV, X rays are cost effective and easier to implement when compared with other random mutagenesis methods. Also, it is less lethal when compared with chemical mutagens, and random UV mutagenesis has improved the thermostability of most of the modified strains [12]. Advancements in geneticand metabolic engineering have made almost any form of hybrid possible with various advantages. Further, with the aid of molecular biology, two or more desirable qualities from different organisms can be expressed in a single organism. These advancements reduce production cost and ease the process [13].Being a disinfectant, producing ethanol through microbial fermentation is a crucial task. Ethanol produced becomes a toxic substance to microbes. Hence, strain development towards ethanol tolerance is a significant area of research in industrial microbiology. In general, strain is modified using random mutagenesis technique. This is done as an attempt to enhance ethanol tolerance and productivity [14]. From the literature, UV has been proved to be apotential mutagen for strain modification, and *Z. mobilis* has not been attempted for random UV mutagenesis using cassava stem as a substrate. Thus, the objective of the work is to perform random UV mutagenesis on *Z. mobilis* to produce enhanced bioethanol- from the hydrolysate of cassava stem pretreated by soaking in aqueous ammonia method.

2. Materials And Methods

2.1. Materials

Cassava stem was collected from the agricultural fields of Namakkal district, Tamil Nadu, India. Yeast extract, glucose, magnesium chloride, ammonium sulphate and potassium dihydrogen sulphate were sourced from HiMedia laboratories, Mumbai, India. Distilled water was made use of in the experiments.

2.2. Collection and Preservation of *Zymomonas mobilis*

Zymomonas mobilis MTCC 92 was procured from microbial type culture collection (MTCC), Institute of microbial technology (IMTECH), Chandigarh, India. The culture was preserved under media of yeast extract glucose salt agar growth media. The medium was prepared adding 10 g of yeast extract and agar of 15g in 900 mL of distilled water. The medium was then autoclaved at 121 °C for 15 min and 100 mL of sterilized 20% (w/v) glucose solution was added. Finally, 10 mL each of stock solutions (Magnesium chloride, ammonium sulphate and potassium dihydrogen phosphate, each at concentration of 100 g/L) was added. The medium was stored at 30 °C and the original culture was transferred to a fresh plate once in two weeks.

2.3. Preparation of Cassava Stem Hydrolysate

Cassava stem hydrolysate was prepared by pretreatment with soaking in aqueous ammonia (SAA) method. The pretreatment parameters were optimized following the procedure reported in the literature [11]. The hydrolysate from the SAA pretreatment of cassava stem was used in the research for microbial fermentation.

2.4. Random UV Mutagenesis

Z. mobilis (wild strain) was sub-cultured using nutrient broth. This culture was poured in a Petriplate during the log phase of growth. The plate was subjected to a random UV mutagenesis by placing it under UV light at 60 cm for 60 min.

2.5. Batch Fermentation Studies

For Bioconversion, the inoculum and the substrate were provided necessary environmental conditions in a 250mL Erlenmeyer flasks to carry out the batch fermentation studies.The flasks were placed on a thermostat incubated shaker by varying pH (3 - 11), substrate concentration (2 -10 g/L), inoculum size (2 - 10% (v/v)), temperature (27– 47 °C), agitation speed (0 - 100 rpm), and time (12 – 120 h) which were optimised for native and modified strains. Ethanol concentration was measured from the supernatant. Triplicates were done for all experiments and the mean value was taken as a response.

2.5.1 Effect of pH on Ethanol Production

During fermentation, the pH effectwas studied with different values ranging from 3 to 11 with the interval of 2 while substrate concentration was fixed at10 g/L and the temperature was maintained 37°C, with fixed inoculum size of 10% (v/v) and constant agitation speed of 100 rpm for 120 h.

2.5.2. Effect of Substrate Concentration on Ethanol Production

The effect of substrate concentration was analysed by modifying the substrate concentration from 2 to 10 g/L while other parameters were fixed as follows,pH at 5, temperature at 37°C, inoculum size at 10% (v/v) and agitation speed at 100 rpm for 120 h in single-step fermentation.

2.5.3. Effect of Inoculum size on Ethanol Production

The fermentation media was prepared by replacing glucose with cassava stem hydrolysate in growth media. By varying size of inoculum from 2 to 10% (v/v) and maintaining others parameters such as substrate concentration of 10 g/L, pH of 5, temperature of 37 °C and agitation of 100 rpm for 120 h, the effect of inoculum on single-step fermentation of *Z.mobilis* was studied.

2.5.4. Effect of Temperature on Ethanol Production

The temperature of fermentation was adjusted by thermostat shaker (Scigenics (India) Private Limited, Chennai, India). The effect of temperature was studied by varying temperature from 27 to 47°C in a single-step fermentation, while fixing other parameters such as substrate concentration at10 g/L, 5 pH, inoculum size at 10% (v/v) and agitation at 100 rpm for 120 h.

2.5.5. Effect of Agitation Speed on Ethanol Production

The hydrolysate of SAA pretreated cassava stem was added at a concentration of 10 g/L. It was inoculated with 10% (v/v) of inoculum for 120 h at a constant temperature of 37°C on shaker varying speed from 0 to 100 rpm for bioconversion studies while the pH of the medium was maintained at 5.

2.5.6. Effect of Fermentation Time on Ethanol Production

Single-step fermentation was studied by varying fermentation time from 12 to 120 h to observe the change in ethanol concentration while maintaining constant substrate concentration of 10 g/L, temperature of 37°C,5 pH, inoculum size of 10% (v/v) and agitation of 100 rpm.

2.6. Kinetics and Thermodynamics of Ethanol Fermentation

Ethanol productivity is calculated as the ratio between ethanol concentration and fermentation time. Specific ethanol production rate is computed as the ratio between ethanol productivity and initial substrate concentration.

Kinetic studies were studied through Michaelis-Menten and rate law mechanisms. Michaelis-Menten equation is given as in Equation (1):

$$v = \frac{V_m . C_s}{K_m + C_s} \quad (1)$$

where v is ethanol productivity, v_m is maximum ethanol productivity and C_s is substrate concentration.

Rate law equation is given as in Equation (2):

$$\left(\frac{-dC_s}{dt}\right) = k.C_s^n \quad (2)$$

where C_s is substrate concentration, t is fermentation time, n is reaction order and k is rate constant.

Arrhenius model as given in Equation (3) is used to study the thermodynamics of ethanol fermentation. Specific ethanol production rate (q_p) is employed to estimate thermodynamic parameters enthalpy,entropy and Gibbs free energy using Equations (3) and (4):

$$q_p = T\left(\frac{k_B}{h}\right) e^{\Delta S^\circ/R} e^{-\Delta H^\circ/RT} \quad (3)$$

$$\Delta G = \Delta H - T.\Delta S \quad (4)$$

where, T, k_B, h, R, ΔH, ΔS and ΔG are absolute temperature (K), Boltzmann constant ($1.38x10^{-23}$ J/K), Planck's constant (6.626×10^{-34} J.s), gas constant (8.314 J/mol.K), enthalpy of activation, entropy of activation and Gibbs free energy change, respectively.

Non-linear forms of Michaelis-Menten, rate law and Arrhenius equations are solved using Microsoft Excel add-in program, Solver. Solver is used to calculate the maximum or minimum value of one cell by changing other cells. In this study, sum of square of error was minimized by varying constant parameters to evaluate v_m and K_m, k and n, and ΔS and ΔH in Michaelis-Menten, rate law and Arrhenius equations, respectively. Error function, sum of square of error, is used to determine the deviation between experimental and predicted values.

2.7. Analytical Methods

The concentration of total reducing sugars wasestimated in the broth after centrifuging at 5000 rpm for 10 min by assaying using 3,5-dinitrosalicylic acid method[38]. The concentration of ethanol produced was quantified using dichromate method[39].

3. Results and Discussion

3.1. Batch Fermentation Studies

3.1.1. Effect of pH on Ethanol Production

From Figure 1, it was clear that ethanol production was higher in the mutant than in native strain atacidic, neutral and basic regions. At pH of 5, maximum ethanol concentration of 1.8 and 2.6 g/L were obtained for native and modified strains of *Z. mobilis* MTCC 92, respectively.Ethanol production is enhanced during acidic regime than alkaline solution. The results show that mutation has induced ethanol tolerance in the strain [40]. Also, decrease in ethanol concentrationafter neutral region show that random mutation was not in favour of production for both strains.

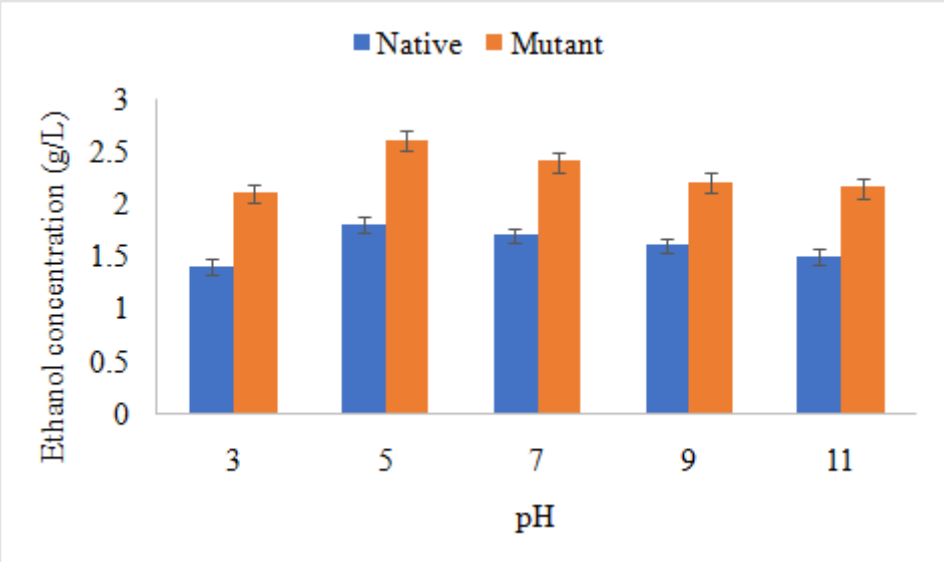

Figure1. *Effect of pH on ethanol concentration for native and modified strains (Substrate concentration = 10 g/L, temperature = 37 ℃, inoculum size = 10% (v/v), agitation speed = 100 rpm and fermentation time = 120 h)*

3.1.2. Effect of Substrate Concentration on Ethanol Production

From Figure 2, it was clear that ethanol production was higher in the mutant than in native strain even at high substrate loading. At substrate concentration of 8 g/L, ethanol concentration reached saturation point after which it formed a plateau. A maximum ethanol concentration of 1.65 and 2.6 g/L were obtained for native and modified strains of *Z. mobilis* MTCC 92, respectively, from the effect of substrate loading from 2 to 10 g/L. Ethanol production is enhanced with an increase in substrate loading. The results show that mutation has induced substrate uptake in the strain[42]. Also, plateau in ethanol concentration after saturation point at 8 g/L show that random mutation was not in favour of production for both strains.

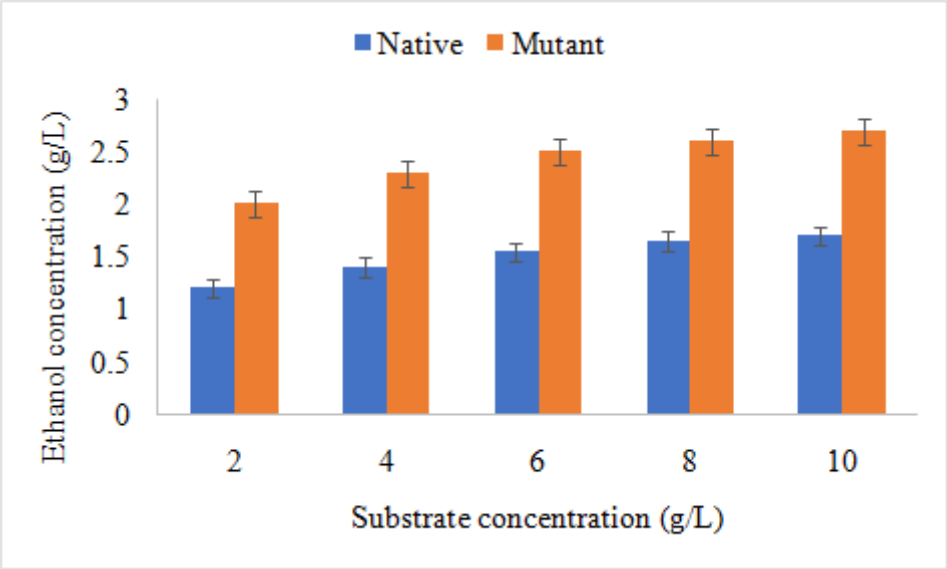

Figure2. *Effect of substrate loading on ethanol concentration for native and modified strains (pH = 5, temperature = 37 ℃, inoculum size = 10% (v/v), agitation speed = 100 rpm and fermentation time = 120 h)*

3.1.3. Effect of Inoculum size on Ethanol Production

From Figure 3, it was observed that ethanol production was higher in the mutant at inoculum size from 2 to 10% (v/v). But the yield of native and mutant strains decreased after 8% (v/v) inoculum size. The results show that mutation had conferred favourable ethanol production at lower inoculum size[42]. The maximum ethanol concentrations of 1.75 and 2.75 g/L were achieved at 8% (v/v) for both native and modified strains. This shows that inoculum size had played a crucial role in ethanol production.

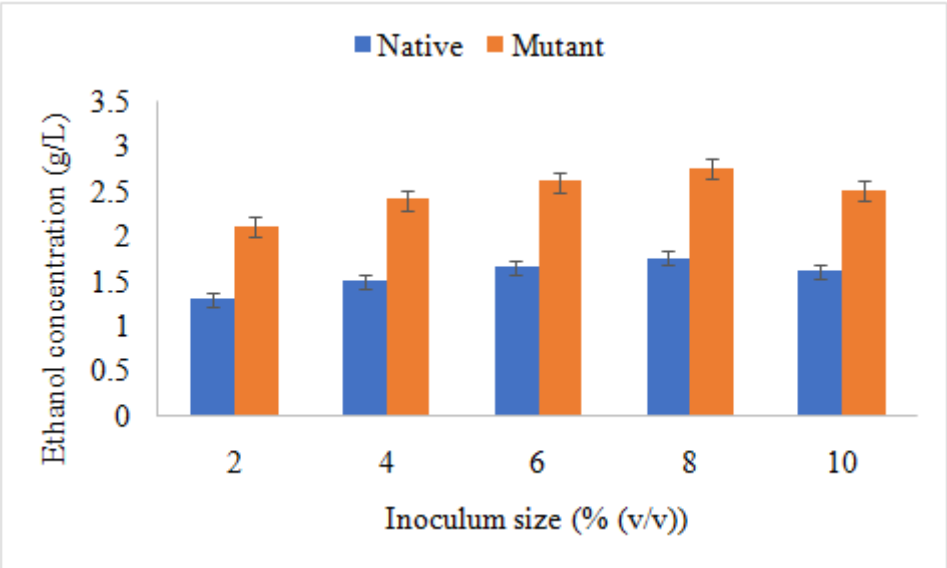

Figure3. *Effect of inoculum size on ethanol concentration for native and modified strains (Substrate concentration = 8 g/L, temperature = 37 ℃, pH= 5, agitation speed = 100 rpm and fermentation time = 120 h)*

3.1.4. Effect of Temperature on Ethanol Production

From Figure 4, it was observed that ethanol production was higher in the mutant at higher temperature. The ethanol yield of mutant gradually increased as the temperature increased. The results show that mutation had conferred toleranceto higher temperature for mutant, which has enhanced the thermal stability of the native strain. It is a desirable quality for ethanol fermentation. The maximum ethanol concentrations of 1.85 and 2.65 g/L were achieved at 37°C for native and modified strains. Mutated strain has produced insignificant ethanol after 37 °C, but, for native strain, ethanol production is reduced.

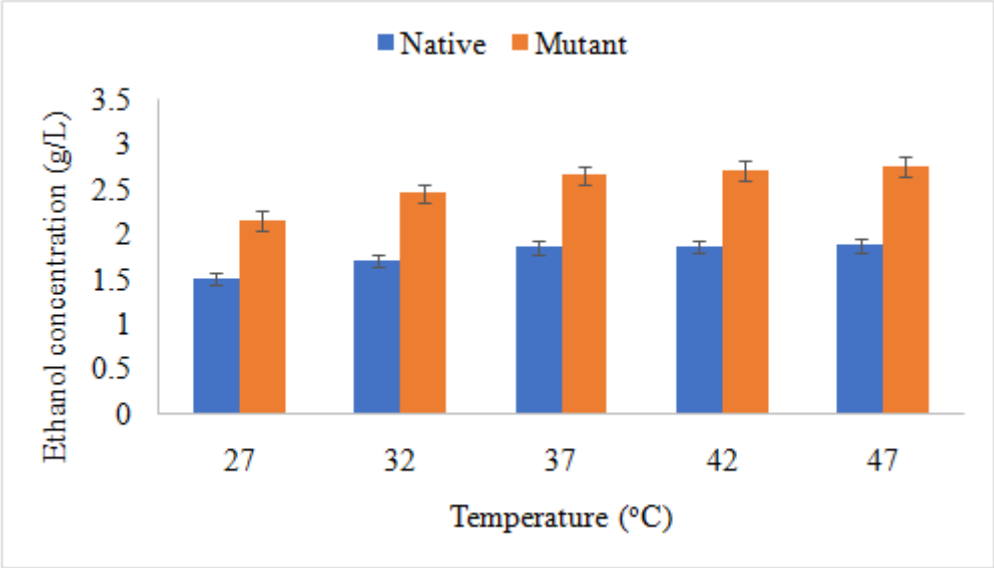

Figure4. *Effect of temperature on ethanol concentration for native and modified strains (Substrate concentration = 8 g/L, pH = 5, inoculum size = 8% (v/v), agitation speed = 100 rpm and fermentation time = 120 h)*

3.1.5. Effect of Agitation Speed on Ethanol Production

From Figure 5, it was observed that the effect of static and dynamic conditions was studied on ethanol production by varying agitation speed fromzero to 100 rpm. But the yield of mutant increased at static to 25, 50 and 75 rpm. This shows that mutation had conferred increased mass transfer effects between medium and mutated strain at higher agitation speed [43]. The maximum ethanol concentration of 1.9 and 2.8 g/L for native and mutated strains, respectively, was achieved at 75 rpm.

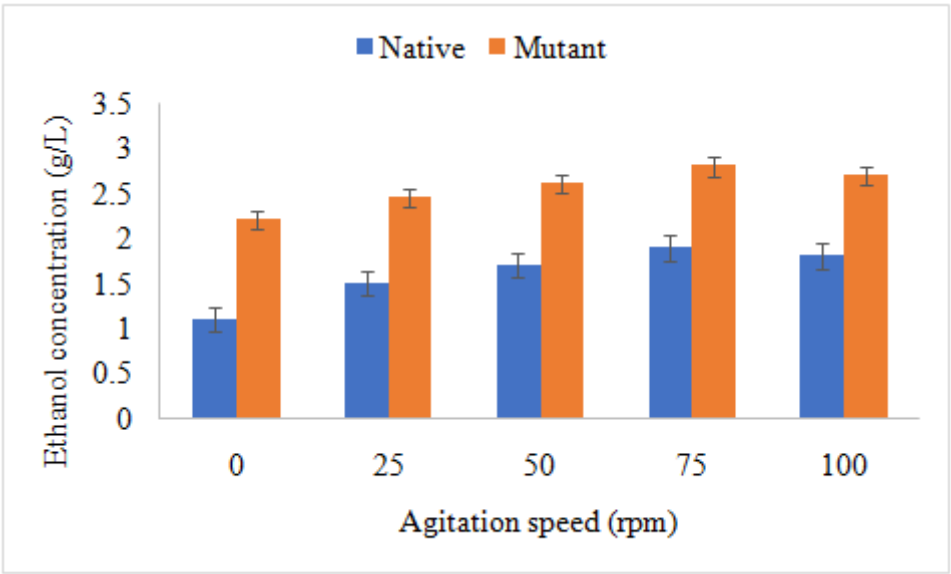

Figure5. *Effect of agitation speed on ethanol concentration for native and modified strains (Substrate concentration = 8 g/L, temperature = 37 ℃, inoculum size = 8% (v/v), pH = 5 and fermentation time = 120 h)*

3.1.6. Effect of Fermentation Time on Ethanol Production

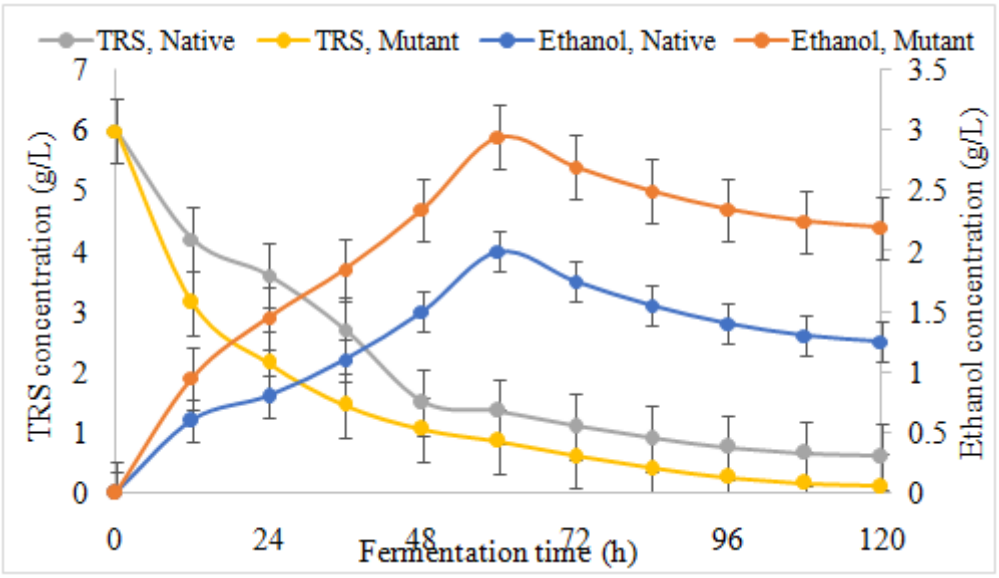

Figure6. *Effect of fermentation time on ethanol concentration for native and modified strains (Substrate concentration = 8 g/L, temperature = 37 ℃, inoculum size = 8% (v/v), pH = 5 and agitation speed = 75 rpm)*

Figure 6 shows the sugar consumption and ethanol production profile. During fermentation, total reducing sugars is converted to ethanol by fermentative enzymes. After 60 h, a decrease in ethanol production is observed due to the formation of inhibitors. In the present study, the maximum product yield coefficient was found to be 0.295 g ethanol per g cassava stem and 0.49 g ethanol per g total reducing sugars for mutated strain, against 0.0.2 g ethanol per g cassava stem and 0.33 g ethanol per g total reducing sugars.

2.6. Kinetics and Thermodynamics of Ethanol Fermentation

Table1. *Kinetic and thermodynamic parameters of ethanol fermentation for native and modified strains*

	Parameters	**Native strain**	**Mutated strain**
Michaelis-Menten equation	v_m (g/L.h)	0.0155	0.0240
	K_m (g/L)	1.1289	0.2937
Rate law equation	k (h^{-1})	0.0270	0.0606
	n	1	1
Arrhenius equation	ΔH (J/mol)	5979.76	6905.19
	ΔS (J/mol.K)	957.714	963.765
	ΔG (kJ/mol)	-2140.61	-291.86

Table 1 shows the kinetic and thermodynamic parameters of ethanol fermentation for native and modified strains. A high v_m and low K_m for mutated strain reveal the faster reaction and high affinity for substrate towards active sites of enzymes. Native strain exhibits slower reaction and lower affinity of substrate towards enzymes.Michaelis-Menten assumes that the order of reaction to be unity. So, when the order of reaction to be unity, rate constant of fermentation catalysed by mutated strain shows higher value than native bacteria. The rate constant again proved that the mutated strain catalysed fermentation was faster than native strain.

Thermodynamic parameters reveal the positive values for entropy and enthalpy, and negative value for Gibbs free energy. Negative ΔG° values confirm the viability and spontaneous fermentation from sugar to ethanol, with a strong mutated strain. Increases in temperature can increase thermal tolerance of mutated strain, resulting in an increase in Gibbs free energy for mutated strain. The endothermic process of fermentation confirmed by the positive values of ΔH°. During the fermentation, the positive value of ΔS° designates improved degree of disorderliness.

4. CONCLUSION

The residue after pretreatment is subjected to microbial fermentation. Further *Z. mobilis* culture was mutated by random UV mutagenesis technique at a distance of 60 cm for various time intervals (0 - 60 min). For effective fermentation, the process variables such as pH (3 - 11), time (0 - 24 h), temperature (25 - 44°C), agitation speed (0 - 100 rpm), inoculum size (2 - 10%) and substrate concentration (2 -10 g/L) were optimised for native and modified strain. A maximum ethanol concentration of 17.5 g/L was achieved for modified strain against 13.3 g/L for native strain.

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