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INCREASE IN BIOETHANOL PRODUCTION BY *ZYMOMONAS MOBILIS* BY ADDITION OFZINC IONS IN THE ALCOHOL PRODUCTION MEDIA

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ABSTRACT

It has been reported in literature that *Z. mobilis* has two types of alcohol dehydrogenase gene (ADH), one is Zinc inducible, and another is Iron (Ferrous) inducible. Therefore, it was attempted to increase the alcohol production by inducing the alcohol dehydrogenase activity by addition of Zinc ions. It was standardized that 10 mM Zinc sulphate addition to alcohol producing broth resulted in maximum increase in alcohol production in both shake flask and 2 litre fermentor and it was 1.46 fold (49%) more than the control (using alcohol producing broth without Zinc sulphate). There was 3.2 fold increases in alcohol dehydrogenase activity when it was measured in presence of 10 mM Zinc sulphate as compared to control (ADH activity measured using same cell lysate in the absence of Zinc sulphate).

KEY WORDS: **Bioethanol, Zinc ion, Productivity, ADH Activity**

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INTRODUCTION

In recent years, due to constant increasing emission of greenhouse gasses, major thrust has laid upon production of biofuels instead of using fossil fuels. Bioethanol has been regarded as a favorable alternative energy source, which is both renewable and environmental friendly[1] .Nearly, 73% of the whole ethanol produced globally is used as biofuels [2, 3, 4]. Ethyl alcohol is gaining wide acceptability as fuel as it burns clean and can be quite easily blended with gasoline. *Zymomonas mobilis* is an obligately fermentative bacterium of industrial interest for ethanol production [5,6].This organism ferments glucose to ethanol and carbon dioxide at a level of up to 98% of the theoretical yield (two ethanol molecules per glucose molecule), achieving high concentrations of ethanol that are equivalent to those achieved by *Saccharomyces cerevisiae*. As an alternative organism for the production of ethanol, *Z mobilis* has several advantages over yeast. These are: higher rates of glucose uptake and ethanol production, higher ethanol yields and ethanol tolerance [7,8].This bacterium uses a modified Entner-Doudroff pathway to rapidly produce upto 1·9 mol of ethanol per mol of glucose used. Thus *Z. mobilis* is a promising candidate for large scale production of ethanol [9, 10].

Alcohol dehydrogenase (ADH) converts acetaldehyde (produced from pyruvate by pyruvate decarboxylase) to ethanol. Two isoenzymes of ADH have been identified in *Z. mobilis* [11]. ADHI is a Zinc binding enzyme [12] and appears similar in many respects to the alcohol dehydrogenase of *S. cerevisiae* [13], fungi [14] and plants [15].*Zymomonas mobilis* is an unusual microorganism which utilizes both iron-containing alcohol dehydrogenase (ADHII) and zinc-containing alcohol dehydrogenase (ADHI) isoenzymes during fermentative growth [16]. It is also reported that Zinc ions can induce the activity of alcohol dehydrogenase (ADH) protein in human as this protein has binding sites for Zinc ions [17]. NADH dependant ADH protein catalyses the last step of bioethanol production e.g. the conversion of acetaldehyde to ethanol. If the rate of conversion of acetaldehyde to ethanol is increased, definitely it will increase the overall alcohol production. It was shown that mutants of *E. coli* that overproduced native alcohol dehydrogenase showing the high levels of ethanol [18]. Hence, it can be hypothesized that addition of Zinc ion (in the form of zinc salts) in alcohol producing media will increase the overall alcohol production by enhancing the activity of NADH dependant alcohol dehydrogenase. In this study, we have attempted to examine the effect of Zinc ions on alcohol production by *Zymomonas mobilis* e.g. whether the additions of zinc ions in alcohol production medium can alter the alcohol production by *Zymomonas mobilis*.

MATERIALS AND METHODS

(i)Strains and media

Zymomonas mobilis (MTCC 2427) was purchased from MTCC, Institute of Microbial Technology (IMTECH), Chandigarh and it was used in the present study. YEPD media (20 g/L Dextrose, 20 g/L Bactopeptone and 10 g/L yeast extract powder) was used for propagation of *Z.mobilis*. Stable cultures were maintained in medium containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose with 50% glycerol and stored at - 80^{0}C. Slant culture medium: 20 g/L Glucose, 20 g/L peptone, 10 g/L yeast extract powder and 20 g/L agar.

(ii) Alcohol estimation in culture broth by titration method

Single colony was picked up from YEPD-agar plates and incubated in 50 ml YEPD broth and incubated in rotary shaker at 200 rpm at 30^{0}C. After 48 hrs incubation , 1ml of microbial broth was withdrawn, it was centrifuged to discard cell mass.50µl filtrate was poured in 100ml flask.In the filtrate, 10ml of Potassium dichromate solution was added & kept for overnight incubation. One water control which contained 50µl water (in place of 50 µl culture filtrate) was

taken and 10 ml potassium dichromate solution was also added to it and incubated overnight. Next day, 1ml of Potassium Iodide solution was added in each flask followed by addition of 90ml distilled water and titrated against sodium thiosulphate until straw yellow color developed. Once straw yellow color came out, 1ml of starch indicator was added and titration was continued until solution became colorless.

(iii)Induction of Alcohol production by addition of Zinc Sulphate in Zymomonas mobilis culture broth

To examine the level of induction in alcohol production by addition of Zinc sulphate in alcohol production medium, different concentrations of Zinc Sulphate (from 2 mM to 40 mM) were added to 50ml YEPD broth and an equal amount of inoculum of *Z.mobilis* was added to the broth and grown at 30^0C for 48 hours on rotary shaker.A control containing *Z.mobilis* in YEPD media without Zinc sulphate was also grown at 30^0C for 48 hours on rotary shaker. Amount of alcohol was estimated in all the samples along with control by titration method as stated earlier.

(iv) Production of alcohol in Fermentor with Zymomonas mobilis in fermentation medium with and without 20mM Zinc Sulphate separately

A seed culture in a one-liter Erlenmeyer flask containing 250 ml of YPS growth medium was prepared using a 1.0 ml frozen suspension of *Z.mobilis*. The culture was incubated at 28 °C for 12 hours at 200 rpm in an orbital shaker.Then inoculum was transferred to the Bioage Fermentor containing 1.50 liters of fermentation medium (the fermentor medium contains sucrose: 200 gm/litre, ammonium sulphate: 01gm/litre, Magnesium sulphate: 0.5 gm/litre, potassium dihydrogen phosphate: 01 gm/litre; pH 7.0).The Fermentor was controlled at 30°C and pH 7.0. Aeration rate was set at 2.5 l min^{-1} (0.5 vessel volumes per minute), and agitation speed was 150 rpm. 29% NH_4OH base solution was used to control pH.Dissolved oxygen was controlled at 30% during the cell growth phase for 24 hours followed by an anaerobic production phase for another 48 hours.Amount of alcohol present in fermentation broth was measured by the method of Kaputi et al [19] and gas chromatography.

(v) Estimation of sucrose by Phenol-sulphuric acid method before and after fermentation

Determination of sugars using phenol-sulfuric acid is based on the absorbance at 490 nm of a colored aromatic complex formed between phenol and the carbohydrate.The amount of sugar present is determined by comparison with a calibration curve using a spectrophotometer [20].

(v)Alcohol Dehydrogenase Assays

(a) Preparation of cell lysate of from Zymomonas mobilis [21]

Cells were lysed by mixing each g wet wt. of cell paste (approx0.26g dry wt.) with 6ml of 30mM K_2HPO_4, containing 0.1% Nonidet P-40, 2mM $MgCl_2$ 10 mM-2-mercaptoethanol, 0.15 mg of lysozyme / ml and 3 of microgram deoxyribonuclease I/ml. The pH of the suspension was adjusted to 8.2 with 1M Tris, and the mixture was stirred for 2-3 h at 30°C.The pH was then lowered to 6.0 with 1 M-Mes in 5 M-acetic acid, and the cell debris was removed by centrifugation at 10000-15000g for 15min. Satisfactory extraction was indicated by a protein content of 12+2mg/ml.

(b) ADH Assay [22]

NADH dependant ADH activity was assayed at 25^0C by measuring the decrease in absorption at 340 nm of NADH (Molar Absosptivity=6220 $M^{-1}cm^{-1}$).The reaction mixture consisted of 5.0 micro mole of acetaldehyde, 0.4 micromole of NADH, 75 micromole of pyrophosphate buffer (pH 7.8) and 100 microlitre of enzyme solution in a total volume of 2 ml.

RESULTS

1. Induction of alcohol production by addition of Zinc sulphate in YEPD broth

Zinc sulphate was used as additional component in normal YEPD media components

as it may assist in the induction of alcohol production by augmenting the enzyme activity of alcohol dehydrogenase of *Z.mobilis*. Different concentrations of Zinc sulphate starting from 0(regarded as control) to 30 mM were added to alcohol production medium (50 ml in 250 ml shake flask) and an equal amount of inoculum were added, grown for 48 hours at 30ºC and alcohol amount in 50 microlitre culture supernatant was estimated by titration method. The increase in alcohol production due to the addition of various concentration of Zinc sulphate and related cell mass concentration was presented in table no1.

Table no.1

Alcohol production by Zymomonas mobilis due to addition of various concentration of Zinc sulphate in alcohol production broth and related optical density of culture broth.

Concentration of Zinc sulphate added to alcohol producing media (mM)	% of alcohol produced (v/v)	Optical density of culture broth at 600nm
0(control)	3.23	5.85
2.5	3.54	5.70
5.0	3.85	5.25
7.5	4.12	4.39
10	4.81	3.19
15	3.48	2.24
20	2.62	1.43
30	1.23	0.98

The alcohol production increased gradually due to the addition of Zinc sulphate in alcohol production broth .The maximum increase in alcohol production was obtained when10mM Zinc sulphate was added to alcohol production broth. But above, 10mM Zinc sulphate addition, the increase in alcohol production ceased to take place due to less growth of cell mass.

2.Production of alcohol in Fermentor having fermentation media along with 10 mM Zinc sulphate and estimation of alcohol by Kaputi Method

Fermentation was carried out using the media as described in the materials and methods and 10mM of Zinc sulphate was added to that media, pH of the media was set at 7. After inoculation in the media fermentation was carried out at 30^0 C in aerobic phase for 24 hours followed by anaerobic phase for 48 hours. After 72 hours of fermentation, fractional distillation was carried out to measure the amount of alcohol by Kaputi Method. Cultural supernatant was also sent to Allele Life Science Pvt. Ltd, Noida, Uttar Pradesh, India for measurement of the alcohol by Gas Chromatography method. The amount of alcohol present in two types of broth was measured after fractional distillation by comparing with the standard graph of alcohol estimation and the amount of alcohol present (v/v) in two types of broth was presented in table no. 2.

Table no. 2

O.D. values for estimation of alcohol by Kaputi method after fermentation in presence of 10mM Zinc Sulphate and amount of alcohol

Sample	Absorbance	Amount of alcohol (in %,v/v)
Distilled filtrate from broth (Without Zinc sulphate)	0.54	10.8%
Distillate from Fermentor broth (with 10 mM Zinc Sulphate)	0.81	16.1%

The increase in alcohol production due to the addition of 10mM Zinc sulphate in fermentation media was estimated to be 1.49 fold (49 % increase) when *Zymomonas mobilis* was used as the inoculum (table no.2). The amount of alcohol produced due to the addition of 10mM Zinc sulphate in fermentation media was estimated to be 15.6% by gas chromatographic method.

3. Alcohol Dehydrogenase assay using the cell lysate of Z.mobilis obtained after fermentation

The absorption data for alcohol dehydrogenase assay with cell lysate was presented in table no. 3.On the basis of those absorption data, alcohol dehydrogenase activity was estimated by following the formulae mentioned below.

Table 3
Spectrophometric values of ADH assay with cell lysate of Zymomonas mobilis with and without Zinc Sulphate

Type of Sample	Absorbance at 340 nm
Blank without zinc (all assay components added excluding cell lysate)	0.624
Assay set up without 20 mM zinc (all assay components including cell lysate)	0.479
Blank with 20 mM Zinc sulphate (all assay components added excluding cell lysate)	0.681
Assay set up with 20 mM zinc (all assay components including cell lysate and Zinc sulphate)	0.337

Calculations for determining the activity of NADH dependant Alcohol dehydrogenase activity

$$\text{Units/ml enzyme} = \frac{(\text{Abs. at 340nm/min Blank} - \text{Abs 340nm/minTest})(2)(df)}{(6.22)\,(0.1)}$$

2= Total volume (in milliliters) of assay
df = Dilution factor, 6.22 = Millimolar extinction coefficient of β-NADH at 340 nm, 0.1 = Volume (in milliliter) of enzyme used
According to given formula:

1) Cell lysate without Zinc sulphate:

$$\text{Units/ml enzyme} = \frac{(0.624-0.479) \times 2 \times 0.1}{6.22 \times 0.2}$$

$$= 0.023 \text{ U/ml}$$

2) Cell lysate with Zinc sulphate:

$$\text{Units/ml enzyme} = \frac{(0.681-.337) \times 2 \times 0.1}{6.22 \times 0.2}$$

$$= 0.058 \text{ U/ml}$$

So, the fold increases in ADH activity in presence of Zinc sulphate =.058/.023 = 2.52 fold. The ADH activities were found to be increased by 3.2 fold in presence of Zinc Sulphate by ADH assay.

4. Estimation of reducing sugar before and after fermentation

There is a correlation between sugar utilization and growth of the microorganism and alcohol production[23].Therefore we had estimated the amount residual sugar before and after the fermentation in both cases e.g. with and without induction of 10mM Zinc sulphate (table no.4).

Table no. 4
Estimation of residual Sucrose before and after fermentation

Sample	Amount of sucrose (mg/ml) left	Amount of sucrose (mg/ml) utilized
Before fermentation	0.2	-
After fermentation (w/o Zinc sulphate)	0.525	1.475
After fermentation (with Zinc sulphate)	0.010	1.990

It was observed that there was 1.34 fold increases in sucrose utilization by *Zymomonas mobilis* when 10 mM Zinc sulphate was added in the fermentation broth of alcohol production. This increased sugar utilization had link with the increased alcohol production due to induction by addition of Zinc sulphate.

DISCUSSION

Zymomonas mobilis is reported to have both iron and zinc dependant alcohol dehydrogenase enzyme whereas *Sacchromyces cerevisiae* has only zinc dependant alcohol dehydrogenase. In NADH/NADPH dependent alcohol dehydrogenase, the zinc ion facilitates the binding of acetaldehyde to the active site. Addition of Zinc also stabilizes the structure of ADH like other Zinc metallo protein, thereby increasing its activity. The dissociation of half (2 zinc atoms/monomer) of the total zinc content of the enzyme is associated with the full inhibition of its activity [17].This information indicates that Zinc is essential for the function of ADH as a Zinc metallo protein. Due to the addition of Zinc Sulphate as an additional component in normal YEPD media, the alcohol production increased gradually upto 10 mM Zinc sulphate addition and maximum alcohol production obtained at 10 mM Zinc Sulphate addition (Table no. 1). Therefore, it was established that the addition of Zinc Sulphate can increase alcohol productivity and % of increase was reported to be 49% which can be considered as profitable increase in terms of industrial production. After the standardization of amount of Zinc sulphate to be added in normal YEPD media components in shake flask, the production was carried out in laboratory based fermentor with fermentation media. The increase in alcohol production in fermentor due to addition of 10 mM Zinc sulphate in fermentation media was estimated to be 16.1% (v/v) as compared to 10.8% with normal fermentation media without Zinc Sulphate supplement (table no. 2) and the % increase was also 49% as it was in shake flask. How addition of Zinc sulphate in alcohol production medium resulted in increased alcohol production is a question to be answered. To assess whether addition of Zinc sulphate can induce the activity of Alcohol Dehydrogenase activity or not, Alcohol Dehydrogenase assay was performed with the cell lysate prepared from the *Z.mobilis* cell mass obtained after fermentation. It was shown that alcohol dehydrogenase activity was increased by 3.2 fold in presence of 10mM Zinc sulphate and it can be concluded that increase in alcohol dehydrogenase activity may attribute to alcohol production. It was estimated that there was 1.34 fold increases in sucrose utilization by *Zymomonas mobilis* when 10 mM Zinc sulphate was added in the fermentation broth of alcohol production (table no. 4). This increased sugar utilization had direct correlation with the increased alcohol production due to induction by addition of Zinc sulphate

CONCLUSION

In case of *Z.mobilis*, addition of Zinc ion in alcohol producing fermentation broth can result in increase in the productivity of alcohol, It was estimated that addition of 10mM of Zinc sulphate in fermentation broth led to 49% increase in alcohol production.

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