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Effect of Zinc oxide Nanoparticles on Hyacinth's Fermentation

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Abstract: In this study bio-hydrogen and bioethanol were produced from dry biomass of water hyacinth by microbial fermentation under influence of zinc oxide nanoparticles. For fermentative bio-hydrogen production biomass was first pretreated and then saccharified into fermentable sugars by enzymes. Sugars of enzymatic hydrolysis were xylose and glucose with concentration of 9.0% and 8.0% respectively. For bioethanol production dry plant was saccharified. The reducing sugar obtained in this method containing 5% glucose. The effect of zinc oxide nanoparticles on fermentative hydrogen and ethanol production from water hyacinth biomass was investigated in batch tests by mixed culture and yeast Saccharomyces cerevisiae respectively. Results showed that the specific concentration of zinc oxide nanoparticles decreased the hydrogen yield. Ethanol yield was enhanced by zinc oxide nanoparticles by using it in certain concentration range during fermentation. The maximum ethanol yield of 0.0193g/g of dry weight plant biomass was obtained at concentration of 5 mg/L of zinc oxide nanoparticles. The ethanol yield constitutes 75.68% of the maximum theoretical yield at zinc oxide nanoparticles.

Key Words: zinc oxide nanoparticles, water hyacinth, saccharomyces cerevisiae, fermentation, clostridium bacillus, biofuel.

INTRODUCTION

Water hyacinth plant can tolerate a wide variation in nutrients, temperature and pH. The optimum temperature is between 25°C and 27.5°C, similarly the optimum pH range is from 6 to 8 [1]. The removal of water hyacinth weed from surface of water bodies requires high cost and labor, so it is better to use the plant as raw material for some valuable purpose such as production of biofuel and biogas. Water hyacinth is a promising plant for production of biofuel (ethanol), biogas (bio-hydrogen) and other valuable products because the plant contains a high amount of cellulose and hemicellulose [1, 2, 3, 4, 5, 6]. The utilization of plant for production of bioethanol and bio-hydrogen will be a great approach for development of biologically clean energy and clean environmental sustainability. The biomass of plant contains about 48% hemicellulose, 18% cellulose and 3.5% lignin [2]. It has significant amount of hemicellulose and very less amount of lignin content and is a suitable biomass for bioethanol production [7].

The process of fermentation is complicated and is affected by many factors. It is affected by such as metal ions, inoculums, substrate concentration, reactor type, pH, temperature, salt concentration, strain of organisms, product concentration (ethanol in case of alcoholic fermentation by yeast), intracellular constituents, membrane composition of microbes, media composition, mode of substrate feeding, osmotic pressure, oxygen availability, nutrients availability etc. In order to improve performance of fermentation, it is necessary to consider and understand the effects of these factors [8]. The most important parameters affecting the process of hydrogen fermentation are four, pH, temperature, organic loading concentration and hydraulic retention time [9]. There are some metabolites such as acetic acid, acetaldehyde and medium chain fatty acids which have toxic effect on yeasts, the principle microbes for alcoholic fermentation [10].

A variety of heterotrophic bacteria can be used to ferment carbohydrate under anaerobic conditions to produce bio-hydrogen but the most widely species used in dark fermentation are spore forming Clostridium, bacillus, thermophilic bacteria and the anaerobic acidogenic sludge [11]. Hydrogen consuming bacteria from hydrogen reactor can be avoided by the heat-treatment of seed sludge [12], low pH operation [13] and by addition of inhibitors [14]. Heat treatment kills hydrogenotrophic methanogen while low pH and inhibitor addition inhibit its growth. Hydrogen producing bacteria should be dominated by

using mixed culture. All these bacteria contain hydrogenase which is a key enzyme catalyzing molecular hydrogen formation by combining proton and electrons in dark fermentation.

Ethanol can be produced by fermentation from biomass which includes grains, grass, wood, indigestible plants, sugarcane juice, poplar trees, straw as well as waste from paper mills or livestock such as cattle dung. The productions of ethanol from feedstock like maize grain, molasses, sorghum grain and sugarcane juice is not economical because these materials are expensive and compete with our food [15, 16]. Cellulosic biomass is the best feed stock for ethanol production because it is renewable and available on earth in large quantities. The selection of cheap and carbohydrate rich raw materials such as weed lignocellulose biomass which contain cellulose (20–50%), hemicellulose (20–35%) and polyphenolic lignin (10–35%) is an alternative feed stock for bioethanol production [17, 18, 19, 20]. Lignocelluloses of water hyacinth are of great interest for fermentative ethanol production due to their availability, abundance and relatively low cost [1]. The conversion of biomass (lignocellulose etc.) into bio-ethanol occurs by several processes. (1) The pretreatment of lignocellulose by alkali or acid to remove lignin part because the microbes cannot ferment lignin. (2) Acid or enzymatic hydrolysis to break down cellulose and hemicellulose into simpler sugars, glucose. (3) Microbial fermentation by yeast or bacteria to produce bio-ethanol (4) Distillation of fermentation products to isolate ethanol.

Common baker's yeast, Saccharomyces cerevisiae has long been used for ethanol fermentation. They have the highest rate of sugar conversion into ethanol of all the yeasts in nature and can readily convert glucose or sucrose molecules into ethanol. The optimum temperature for ethanol fermentation by yeast is 26°C to 35°C and ideal pH is 4.5 [21]. Bio-hydrogen and bioethanol production requires essential micronutrients for bacterial and yeast metabolism respectively during fermentation. Their production can be affected by essential trace elements such as iron, zinc, sodium and magnesium [22, 23]. Zinc nanoparticles have unlimited applications in manufacturing of commercial and personal products, food additives as well as in coating and paints [24]. It is one of the most common NPs which is used in personal care products, paper, plastics and building materials, cosmetics, electronics, medicines and military applications due to its unique optical properties high stability, anticorrosion and photo catalytic properties [25]. Zinc NPs are also used in transparent UV-protection film, chemical sensors [26] and as UV-filters in sunscreens [27]. Zinc NPs are released into environment from metal industries, mining and phosphate fertilizer plants they can also enter into aquatic environment through waste water at industrial sites. The Zn NPs enter via domestic sewage due to their extensive production, consumption and releasing rises concern about environmental impacts. Zinc is an essential trace element for living organisms but its nano scale use and high concentration can produce cellular damage [28]. The toxicity of zinc NPs have been documented in literature [29]. The accumulation of zinc NPs in ecosystem is a threat to non-specific target organisms such as bacteria and yeasts. The effect of zinc like above on bacteria and fungi has been demonstrated by various authors [29, 30]. Some studies have reported the toxicity of Zn NPs in bacterial system and vertebrates [31]. The effect of Zn NPs has been evaluated in E. coli [32], B. subtilis [33] and S. aureus [34]. Zn NPs demonstrates significant growth retardation in broad spectrum of bacteria [35].

The release of Zn ions from zinc oxide NPs may be a key factor in their toxicity. The toxicity of ZnO NPs to bacteria is due to the dissolving Zn-ions [36]. It has been determined that the concentration of dissolved Zn ions derived from Zn NPs have toxic effect on P. subcapitata [37]. When Zn NPs with 40 nm diameters sized was applied to S. aureus and Escherichia coli, their viability decreased in 24 hrs [38]. Zn NPs has been shown to decrease the activity of Gram-positive bacteria strains [39]. Complete inhibition of growth by Zn NPs has been shown in planktonic, S. aureus [40]. Ninety five percent growth inhibitions have shown in S. aureus when 1mM of 8 nm Zn NPs were applied for 10hrs [35]. Zn NPs decrease growth of Clostridium butyricum and other hydrogen fermenting bacteria present in sewage sludge.

Yeast, Saccharomyces cerevisiae is used in toxicological evolutions of heavy metals, anticancer, drugs and herbicide [41]. It is one of the most important unicellular organisms, used in molecular and cell biology. It has many similarities with plant and animal cells [42]. The toxicity of Zn NPs to bacteria and other unicellular organisms has been reported however; the cytotoxicity of NPs to yeast is poorly understood. The effect of Zn NPs has also been reported to investigate methane production during Waste Activated Sludge (WAS) anaerobic digestion [43] and resulted low methane production in presence of Zn NPs. The decrease of methane production may be due to toxic effect of Zn NPs on micro-organisms present in WAS. Some studies indicated that the toxicity of Zn NPs on reduction of methane is due to release of zinc ions from NPs [22, 37, 44] still some studies reported that the toxicity is due to Zn NPs themselves rather than zinc ions [44].

To the best of our knowledge the exact mechanism of Zn NPs toxicity is not explored. There are many studies suggesting different mechanism behind its toxic effects. Oxidative stress and lipid per oxidation are adverse toxic effects in many bacteria and yeast which lead to DNA damage, cell membrane disruption and subsequent cell death [45]. Zinc oxide NPs are reported to damage membrane architecture which leads to alter permeability of membrane and subsequently the NPs accumulate in the cytoplasm [46]. Oxidative stress produces by Zn NPs cause loss of cell viability. This is due to high level of

intracellular reactive oxygen species which is toxic to cytoplasmic lipids, proteins and other intermediates present in cells [44, 47]. Some studies indicate that Zn NPs interact with macromolecules such as DNA.

Main aim and objective of this study was to utilize contaminated plant biomass as a source of energy and to assess whether plant loaded with nanoparticles can produce bioethanol and bio-hydrogen. To investigate the effects of zinc oxide nanoparticle's concentrations on ethanol and hydrogen production from water hyacinth biomass. We used yeast, Saccharomyces cerevisiae and mixed culture as fermentative microbes respectively. The ability of water hyacinth biomass, contaminated with nanoparticles for bioethanol and bio-hydrogen production was noted.

MATERIALS AND METHODS

All chemicals used in this study were of analytical grade and their detail is already described by Zada, et al., [1]. Water hyacinth fresh plants were collected from natural ponds of Taxila, situated at the Punjab province of Pakistan [48]. Samples were prepared both for ethanol and hydrogen fermentation. The fermentative organisms (yeast and bacteria here) cannot ferment complex carbohydrates into fermentable products so the plant was first hydrolyzed into simpler sugars, glucose, xylose etc. Two methods were carried out for hydrolysis of complex cellulose, hemicellulose and lignin into simpler units for the process of fermentation.

1- Enzymatic Hydrolysis
2- Acid Hydrolysis

Acid hydrolysis is economical over enzymatic hydrolysis, however, during acid hydrolysis toxic substances are produced which hinder microbial fermentation. Enzymatically plant materials were hydrolyzed with cellulase in two steps process, pretreatment and incubation. The lignin part of carbohydrates hinders enzyme attack, so pretreatment is required to make surface area accessible for enzymes utilization. Pretreatment of water hyacinth was carried out to destroy and remove solid lignin part of the biomass which surrounds cellulose and hemicellulose tightly. For this purpose 3% NaOH solution was prepared. Six grams of plant powder (leaves, stem and roots) were taken and mixed with 100 ml of 3% NaOH solution. The mixture was shaken on hot plate at 50°C with a rotation speed of 150 rpm for 24 hours. After the pretreatment the pH was adjusted to 4.5 with 6M HCl solution[1]. Then the samples were washed with tape water using a $38mm^{-1}$ mesh sieve until the pH value of the drained water reached neutral. After washing the sample was dried at 60°C.

Enzymatic saccharification of plant biomass was performed with commercially available cellulase. Enzymatic saccharification of pretreated hyacinth sample to hydrolyze cellulose and hemicellulose into fermentable sugars was carried out in 250 mL flasks. Five grams of pretreated plant materials was taken in each flask and were autoclaved at 121°C for 20 minutes. Then 50 mL of filter-sterilized commercial cellulase enzyme (Sumitime C; Shin Nihon Chemical Co. Ltd., Japan) solution (cellulase activity: 20 Filter paper units (FPU) (g substrate), (xylanase activity: 615 unit (g substrate) in 0.1M sodium phosphate (pH 4.8) was added to each flask and heated at 45°C for 24hrs with rotation at rate of 150 rpm. Saccharification efficiency was calculated as percentage conversion of pretreated biomass to reducing sugars.

Acid hydrolysis of water hyacinth was carried without pretreatment. 50 mL of 1% sulfuric acid solution was taken in 100 mL flask and 3g of the plant powder was added to it. Flask was autoclaved at 121°C, 15 lbs for 1.5hrs. After this the sample was cooled in tape water and filtered through filter paper. The filtrate was collected, neutralized with 1M NaOH solutions. It was re-filtered through Wattman No. 1 filter paper in order to remove any un-hydrolyzed material. The filtrate was collected and subjected to analyze sugars contents. In second method the plant dry powder was taken in flask and 2% sulfuric acid solution was added to it. The flask was refluxed at 110°C for 5hrs. After heating it was cooled down and filtered. The filtrate was detoxified and analyzed for measuring of reducing sugars. Both methods were compared for best hydrolysis of plant for reducing sugars. Hydrolysate obtained was detoxified and concentrated by evaporation. It was heated to 100°C for 15 min to remove or reduce concentration of volatile components. Any loss in volume during boiling was replaced with heated distilled water. The acid hydrolysate was then saturated with slow addition of solid $Ca(OH)_2$ up to pH 10.0, in combination with 0.1% sodium sulfite. The precipitate, CaSO4 formed was removed by filtration through a 0.45 micron membrane and re-acidified to pH 6.0 ± 0.2 with 1N sulfuric acid. The composition of the acid hydrolysate was analyzed and solution was stored at 10°C for further use.

Bio-hydrogen was produced by fermentation using water hyacinth's hydrolysate and mixed culture. Mixed culture was used for hydrogen fermentation[1]. The mixed culture was composed of anaerobic digested sludge, sludge from sewage water, soil from wheat field, cow dung and lake sediment. Culture was saturated by Clostridium butyricum to enhance the production of hydrogen. Clostridium butyricum TISTR 1032 was grown in biochemistry lab of our University. The microbes were

grown in a medium at 35°C under anaerobic condition for 10 h and stored at 4°C as a stock culture. Clostridium butyricum was activated by mixing 1 mL of stock culture with 10 mL of fresh tryptone sucrose yeast (TSY) extract medium in serum bottle. TSY used, contained 5.0 g tryptone; 3.0 g sucrose; 5.0 g yeast extract and 1.0 g K_2HPO_4 per liter. Argon gas was flushed into serum bottle to ensure anaerobic condition. Medium was incubated at 37°C for 12hrs at 150 rpm on shaker. After first round of incubation it was further enriched by inoculation of fresh TSY and used as inoculum. Heat-shock pretreatment method was used in this study to enriched hydrogen producing bacteria. Pretreatment was conducted in a sterilized pot. In this method whole mixture was heated at 121°C for 20 min.

Nutrition medium for enrichment of microorganism was prepared, 1L of which contained NH_4HCO_3, 7540 mg; K_2HPO_4, 250 mg; Na_2CO_3, 4000 mg; $CuSO_4.5H_2O$, 10 mg; $MgCl_2.6H_2$; 200 mg; MnSO4.4H2O, 30 mg; $FeSO_4.7H_2O$, 50 mg; NaCl, 0.1 g; $CaCl_2$, 0.01 g; $NaMoO_4.2H_2O$, 0.01 g; $Na_2S.9H_2O$, 0.25 g; $CoCl_2.6H_2O$, 0.250 mg and yeast extract, 2.0 g. Medium was autoclaved for removal of contamination before using in fermentation[1].

Dark-fermentation for hydrogen production was performed in 120 mL serum bottles in batch tests. Into each serum bottle, 40 mL of inoculum, 40 mL of autoclaved nutrition medium and enzymatically hydrolyzed plant hydrolysate obtained from 3 g of plant biomass was added. Total fermentation liquor volume of each bottle was adjusted to 100 mL with deionized water. Air was removed by passing argon gas for 3 min from each bottle to ensure anaerobic conditions. Serum bottles were capped with rubber stopper, into which 60 mL syringes were inserted to collect total biogas and placed in reciprocal shaker. Zinc oxide nanoparticles concentration ranged from 0 to 100 mg/L in batch test to check its effects. Zero mg/L concentration of nanoparticles was taken as control. Separate bottles were used for each nanoparticle concentration. Initial pH value of medium was adjusted with 3N NaOH or 3N HCl solutions. Batch experiments were carried out at 35°C in dark room for 4 days. All batch tests were repeated in triplicate. Water hyacinth hydrolysate was used as substrate for bioethanol production. Common yeast, Saccharomyces cerevisiae were used for fermentative ethanol production.

Inoculation medium was prepared in 250 mL conical flasks with distilled, deionized water. Table 1 outline compounds, their amount and concentration used for making the medium. Flasks were capped with rubber stopper and placed in autoclave at 121°C for 15 minutes in order to prevent any microorganism other than the growth of yeast. After sterilization, one loop of live yeast's cells (Saccharomyces cerevisiae) was added into each flask. The flasks were again capped with rubber stoppers and incubated at 30°C for 24hrs at about 150 rpm shaking. Now inoculum was used for fermentation medium[1].

Table 1: Inoculation medium

Compound	Used amount/Concentration
Glucose	1 g / (20 g/l)
Peptone	1 g / (20 g/l)
Yeast extract	0.5 g / (10 g/l)

Supplementary nutrition medium for fermentation was prepared using distilled, deionized water and acetate buffer. Compounds and their subsequent concentration used for nutrition medium are tabulated in Table 2.

Table 2: Nutrition Medium

Compound	Concentration (g/l)
Ammonium Chloride (NH_4Cl)	5.0
Magnesium Sulfate ($MgSO_4.7H_2O$)	1.0
Potassium Dihydrogen Phosphate (KH_2PO_4)	2.0
Sodium Molybdate (Na_2MoO_4)	0.0002
Ammonium Sulfate ($(NH_4)_2SO_4$)	1.0
Copper Sulfate ($CuSO_4$)	0.004
Manganese Sulfate ($MnSO_4$)	0.002
Iron Sulfate ($FeSO_4$)	0.004

To make fermentation medium for ethanol production, hydrolysate was concentrated to 5% (w/v) reducing sugar. Refluxed acid hydrolysate of dry powdered plant was used as fermenting substrate. Ethanol fermentation was conducted in 250 ml Erlenmeyer flasks with working volume of 100 ml. Fermentation medium in each flask contained concentrated hydrolysate of 20 g powdered plant biomass, 4.0 ml supplementary nutrition solution (composition mentioned in Table 2), 2.0 ml yeast inoculum and zinc oxide nanoparticles was in range of 0 to 140 mg/l. For each concentration separate flask was used during fermentation. Control medium (without NPs) was prepared[1].

Initial pH of these media was adjusted at 4.5 with 1N NaOH or 1N HCl solution. Before addition of inoculum, media were sterilized at 121°C for 15 min. Then 4.0 ml fresh inoculum was added into each flask. Carbon dioxide gas was flushed into each flask for evacuation of oxygen to ensure anaerobic environment. A U-shaped tube was capped on each flask and tight with Paraffin film tape. Flasks were incubated at 30°C for 3 days with shaking at rate of 200 rpm. All experiments were carried out in triplicate. Lignin, cellulose and hemicellulose contents were determined by detergent extraction method [1, 49].

Composition of water hyacinth hydrolysate for total producing sugars, from both enzymatic and acid hydrolysis, was measured by DNS (3, 5-dinitrosalicylic acid) method [50]. A UV/VIS-scanning spectrometer of double beam was used for measuring absorbance. For this purpose 2.0 ml of DNS reagent was taken in a test tube and 1.0 ml hydrolysate was added to it. Blank containing 2.0 ml of DNS and 1.0 ml distilled water was run parallel. Test tube was tightly capped and covered with paraffin film to avoid the loss of liquid. Mixture was heated for 10 minutes at 90°C. A red-brown color developed in the mixture. 1.0 ml of potassium sodium tartrate solution was added to mixture in order to stabilize color. After this, test tube was cooled at room temperature; added 8 ml of distilled water and the absorbance was measured with a spectrometer at 540 nm. The amount of reducing sugar was calculated by adopting the following formula[1].

RSY (%) = (Reducing Sugars Concentrations mg/ml) 50ml x 100%
(Substrate Added, mg)

Total biogas, richer in hydrogen and carbon dioxide produced during fermentation was collected and measured by water displacement method using 2% sulfuric acid and 10% sodium hydroxide containing solution. The biogas evolved during fermentation was noted by volume of water replaced by gas. The volumes of gases were corrected to standard conditions of 25°C and 1 atm. Amount of hydrogen gas in the biogas was analyzed by a gas chromatograph (Model 122, Shanghai, China) equipped with a thermal detector of conductivity and a 2 m column which was stainless and packed with a 5 Å molecular sieves. Operating temperature of column was 40°C, detector 80°C and injector was 50°C. Carrier gas used in analysis was helium at a flow rate of 12 ml per minute. For GC analysis, gas samples were collected through a hypodermic needle. A gas sample of 5 mL was injected to gas chromatograph for analysis. 99.8% pure hydrogen was injected into GC to obtain standard. Other gases produced during fermentation were not detected except hydrogen sulfide. Biogas was also checked for presence of hydrogen sulfide by using another gas chromatograph which was equipped with a flame photometric detector. The capillary column used was HP-5. Soluble metabolites produced during hydrogen fermentation were analyzed. The production of hydrogen is expressed in terms of yield.

For analysis of ethanol and other metabolites a 200-250 µL sample was collected from fermentation media and centrifuged to separate any solid residue and yeast. Then production of ethanol and other metabolites during fermentation was analyzed by using Gas Chromatograph Mass Spectrometer (GC-MS) QP2010 Ultra made in Shimadzu, Japan, fitted with an Agilent DB5MS USA Alcohol capillary column (ID: 0.32 mm, length: 7.5 m, film: 20 µm). Program conditions were as such, temperature program: 125°C, column temperature 125°C, detector temperature 250°C, injector temp 250°C, linear velocity 200 cm/sec, split ratio 20:1, rate 15°C/min, final temperature 150°C. Ethanol was measured in distillates by using an Anton Paar DMA 500 density meter which was calibrated against air (having density 0 mg/ml^3) and boiled deionized water (having density 0.99715 g/cm^3). Reading of each sample was taken at 20°C.

Different methods are used for synthesis of zinc oxide nanoparticles. The main technological difference between various production methods involve process temperature, zinc precursor, unit operation used and pH of solution. Here a typical precipitation method was used for synthesis of zinc oxide nanoparticles. For synthesis of zinc oxide nanoparticles, chemicals used were zinc acetate di-hydrate ($Zn(CH3COO)_2 \cdot 2H_2O$), Octadecylamine ($CH_3(CH_2)_{17}NH_2$) and methanol. All chemicals were of analytical grade and needed no further purification. 0.3M zinc acetate di-hydrate (Zn (CH3COO)2 · 2H2O) and 0.5M octadecylamine (CH3(CH2)17NH2) solutions were prepared and mixed in 100 mL methanol. It was stirred for 30 min in order to complete dissolution. pH of solution was measured and found to be 11.8. The mixture was transferred to refluxing pot after complete dissolution and was refluxed at 65°C for 6hrs. After refluxing mixture for 6hrs white colored precipitate was appeared in mixed solution. Precipitate was washed several times with methanol and dried at room temperature. When the precipitate was dried, it appeared as grown powder. Powder was annealed at 500°C temperature for 1 hr in air and nanoparticles were obtained.

Two experimental techniques, X-ray diffraction (XRD) [1, 51] and Scanning Electron Microscopy (SEM) were used for characterization of zinc oxide nanoparticles. Phase identification of zinc oxide nanoparticles was carried out by X-ray diffraction on a Bruker D8 ADVANCE diffractometer with CuKα radiation (λ = 0.15418 nm). Morphology (shape, size and arrangement of particles) and topography (surface feature) of nanoparticles were characterized by a scanning electron microscope (FEI, NOVA Nano SEM 230). The Scanning Electron Microscopy (SEM) is one that permits the study of composition of biological and physical materials and surface morphology [48].

RESULTS

Water hyacinth was dehydrated before using because it contained high amount of moisture contents (92-94%). Volatile solids measured were organic matter which constitutes 77-79% of total solids present in plant. This data indicated that water hyacinth plant is rich in raw materials for bioconversion into bio-hydrogen, bio-ethanol and other important biofuels like biodiesel etc. The plant also contained considerable amount of crude proteins and fats [48]. Presence of proteins is appreciable to provide nitrogen for bioconversion process. Average composition of plant found is tabulated in Table 3.

Table 3: Average Composition of Water hyacinth

Constituents	Percent of net weight
Moisture	91.4-93.6
Total solids	4.9-7.3
Volatile solids	3.9-5.8
Organic Components	(percent of total solids)
Cellulose	19.2 ± 0.003
Hemicellulose	41.6 ± 0.032
Lignin	4.0 ± 0.003
Crude proteins	12.1 ± 0.012
Crude fats	0.9 ± 0.01

Dilute sulfuric acid hydrolysis under reflux at 110°C for 3hrs was very effective in releasing good amount of fermentable sugars than autoclaving at 121°C for 1.5hr. However, hydrolysis of plant biomass by autoclaving reduces chance of toxic compounds production which may be inhibiting process of fermentation. Moreover, acid hydrolysis by autoclaving solution produce high amount of glucose which is suitable for ethanol fermentation rather than hydrogen fermentation because yeast convert glucose to ethanol by fermentation. The amount of glucose produced after 1.5hr autoclaving using 1% sulfuric acid solution was 2.5 g/50 g (0.05g/g) of dry weight (DW) plant biomass. After 5hrs of reflux with 1% sulfuric acid solution, reducing sugar yield was 10.5 g/50 g (0.21 g/g) of dry biomass of the plant of which D-glucose was 1.0 g, D-galactose 0.8 g, L-arabinose 1.1 g, D-mannose 2.0 g and D-xylose was 5.6 g. In addition to reducing sugars plant hydrolysate also contained small amount of toxic components such as soluble lignin derivatives, furfural and acetic acid which are known to reduce process of fermentation as they are toxic for microorganisms.

Table. 4: Percentage composition of reducing sugars in hydrolysate produced by refluxed and autoclaved hydrolysis

Reducing sugars	Glucose	Galactose	Arabinose	Xylose	Mannose
Method 1: (%)	2.0 ± 0.02	1.6 ± 0.04	2.2 ± 0.07	11.2 ± 0.01	4.0 ± 0.05
Method 2: (%)	5.0 ± 0.03	N.D	N.D	N.D	N.D
Method 1: Refluxed hydrolysis; Method 2: Autoclaved hydrolysis; N.D: Not Detected					

Enzymatic hydrolysis of alkaline (NaOH) pretreated water hyacinth biomass was carried out by commercial enzymes. Reducing sugars produced by this method were used for hydrogen fermentation[1]. Hydrolysis was very effective in generation of reducing sugars for hydrogen production. Pretreatment by NaOH improved hydrolysis of plant biomass. After being autoclaved at 121°C for 20 min the hydrolysate was analyzed for detection of reducing sugars. The sugars yield was 8.5 g/50 g (0.17 g/g) of dry weight plant's biomass after reaction of commercial enzymes for 24 h. Out of 8.5 g of

concentration of glucose was 4.0 g and that of xylose was 4.5 g. Presence of other reducing sugars may be expected but they were not analyzed.

Pretreatment by NaOH was used to destroy lignin component of plant biomass and to release cellulose and hemicellulose which were enzymatically hydrolyzed to reducing sugars for hydrogen fermentation. Actually NaOH breaks hydrogen bond of lignocellulose molecules due to which surface area of cellulose molecules was increased. So high surface area became available for enzymes to hydrolyze cellulose and hemicellulose and produce reducing sugars. High concentration of NaOH increases the efficiency of reducing sugars production and hence increases hydrogen fermentation. However, concentration of NaOH more than 5% was unfavorable for hydrogen producing bacteria and cellulase activities.

Table 5: Percent composition of reducing sugars in hydrolysate produced by enzymatic hydrolysis

Reducing Sugars	**Xylose**	**Glucose**
Percentage Composition	9.0 ± 0.01	8.0 ± 0.03

Table 6: Calculating particle crystalline size of zinc NPs from FWHM of X- ray diffraction pattern

Phase	**Peak position**	**FWHM (in radian)**	**Cos θ**	**Particle size (nm)**
100	31.87◦	0.004016	0.961597733	36.07929
002	34.52◦	0.005908	0.955019944	24.69431
101	36.35◦	0.006520	0.950135459	22.48929
102	47.63◦	0.006729	0.914889221	22.62885
110	56.68◦	0.009408	0.880146162	16.82457
103	62.95◦	0.011777	0.852913627	13.87037
200	66.50◦	0.015073	0.836286155	11.05224
112	68.04◦	0.015133	0.828842326	11.10777
201	69.16◦	0.007681	0.823334533	22.03131
004	72.76◦	0.009810	0.805100890	17.64023

Fermentability of water hyacinth's hydrolysate was increased with detoxification by boiling it at 100°C followed by over-liming with $Ca(OH)_2$ to pH 10.0 in presence of 0.1% sodium sulfite. Boiling removed volatile compounds such as furfural while the over-liming reduced organic acids concentration such as acetic acids. The furfuryl acid is formed from furfural, which can be removed by condensing with other components of hydrolysate [52]. The actual mechanism of over-liming action is still not clear. Over-liming results the loss of small amount of reducing sugars.

X-ray Diffraction (XRD) study of zinc oxide NPs was performed in order to identify the phase and crystalline size of NPs. The XRD pattern of NPs is shown in Fig 1. The pattern showed formation of zinc NPs with hexagonal phase (wurtzite structure). Sharp diffraction peaks as illustrated in the Fig 1 indicate best crystallinity of particles. There was no characteristic peak confirming the absence of any impurity in XRD pattern. Result showed high purity of particles. XRD pattern of NPs showed a series of characteristics peaks at angle (2 θ°) 31.87°, 34.52°, 36.35°, 47.63°, 56.68°, 62.95°, 66.50°, 68.04°, 69.16° and 72.76° which correspond to miller indices (100), (002), (101), (102), (110), (103), (200), (112), (201) and (004) respectively and are in accordance with hexagonal phase ZnO (JCPDS card file No. 361451). The mean crystalline size (D) of zinc NPs was determined using Scherrer equation, $D = 0.9\lambda / (\beta \cos\theta)$, here 0.9 is the machine constant, λ is wavelength (Cu Kα), β is full width at the half-maximum (FWHM) and θ is Bragg diffraction angle. Average crystalline size of zinc oxide NPs calculated was found to be 19.84 nm.

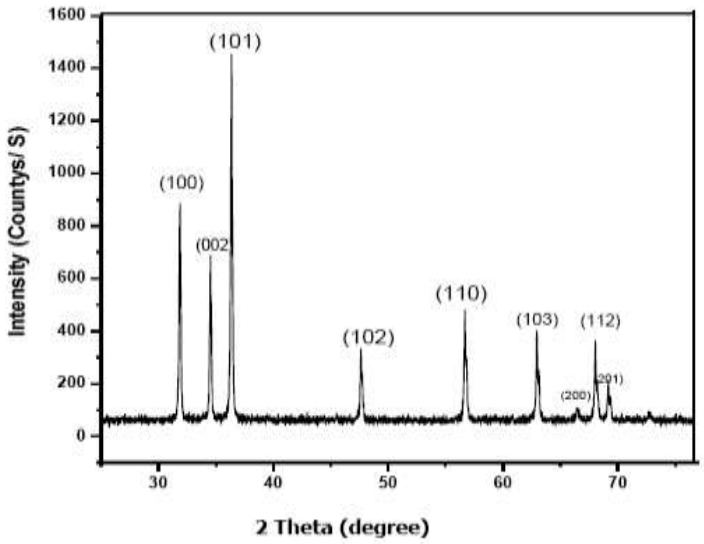

Figure 1: XRD pattern of zinc oxide nanoparticles

Morphology of zinc oxide NPs was studied by scanning electron microscopy (SEM). The SEM image is illustrated in Fig 2. Representative SEM image in Fig 2 indicate that morphology of resultant particles is uniform, regular and spherical sponge like in shape.

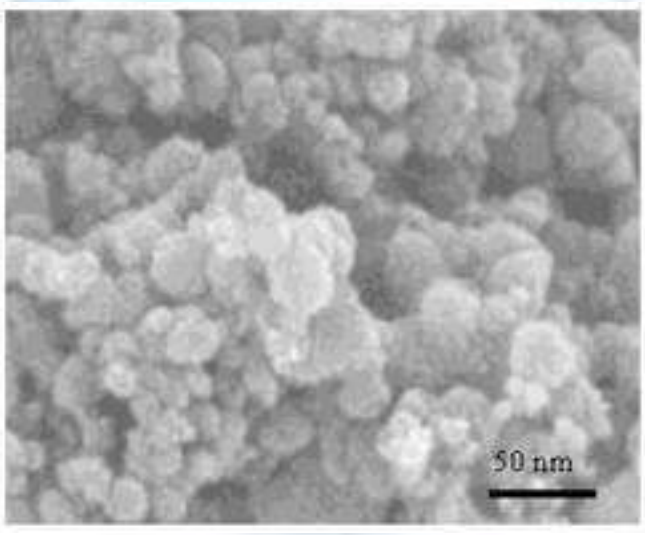

Figure 2: SEM image of zinc oxide nanoparticles.

Fig 7 illustrates effect of zinc NPs on hydrogen yield in batches fermentation. During fermentation initial pH was adjusted at 6.5 and concentration of zinc oxide NPs used was in range of 0 to 100 mg/L. without NPs was taken as control. Individual batch experiments were carried out and each experiment was observed until production of hydrogen from each serum bottle stopped. Biogas produced contained only hydrogen and carbon dioxide in this study. Hydrogen yield was decreased when particles concentration was increasing from 0 to 100 mg/L. This indicated that zinc NPs inhibit hydrogen production. Fig 7 also shows that in range of 20 to 60 mg/L of particles concentration the trend of hydrogen yield was almost same. This suggested that in certain concentration ranges, nanoparticles have same effect on fermentative hydrogen production. With increase in NPs concentration than 10 mg/l, hydrogen yield drastically decreased to 72 mL. At 5 mg/L of zinc oxide NPs the hydrogen yield was similar to control. This indicated that in certain experimental range, particles concentration up to 5 mg/L did not affected hydrogen production.

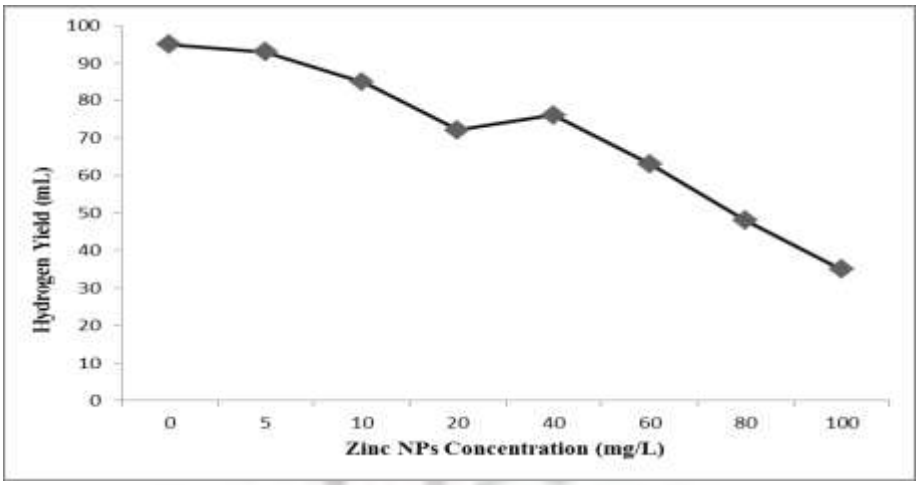

Figure 37: Effect of zinc oxide nanoparticles on hydrogen yield.

Anaerobic hydrogen yield during course of fermentation in control experiment was 95 mL/3 g (31.66 g/g) of plant materials, while at 100 mg/L of NPs (the maximum ZnO NPs concentration used in this study) hydrogen yield was 35 mL/3 g (11g/g) of plant biomass. Compared to control, hydrogen yield was evidently decreased about 58.33% when particles concentration was 100 mg/L. Effect of incubation time on hydrogen yield was investigated under 40 mg/L of zinc oxide NPs concentration. Result obtained was illustrated in Fig 4. The result showed that initially in 12hrs of fermentation period, average rate of hydrogen yield was 9.1 mL/h. After 12hrs, rate of hydrogen yield was drastically decreased. Hydrogen production was completely ceased at 24hrs. The metabolites such as acetic acid, ethanol etc. may be produced during fermentation but their analysis was monitored in this study.

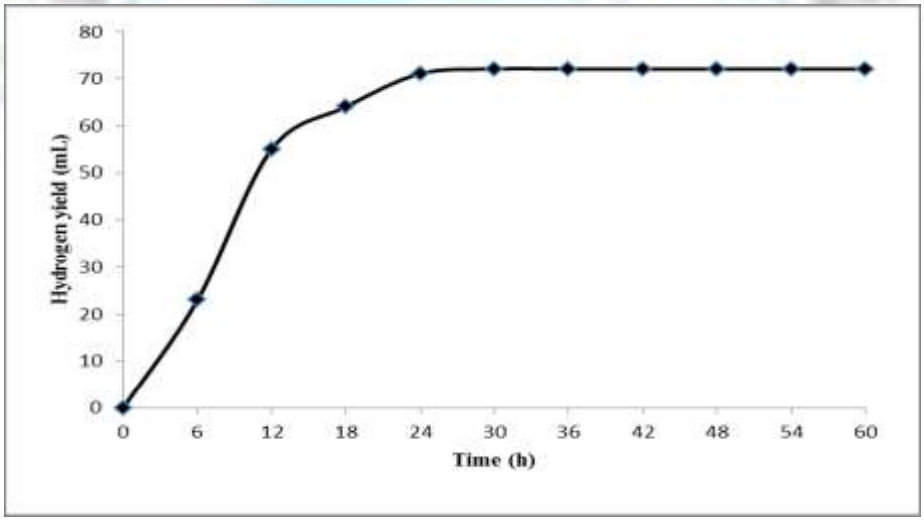

Figure 4: Effect of incubation time on hydrogen yield at 40 mg/L concentration of zinc oxide NPs.

The effect of ZnO NPs concentration on anaerobic fermentative ethanol production by Saccharomyces cerevisiae is illustrated in Fig 5. In this study concentration of ZnO NPs was adjusted in range of 0, 2, 5, 10, 20, 40, 80, 120 and 140 mg/l. For each concentration separate Erlenmeyer flask was used. Initial pH of fermentation media were adjusted at 4.5 with NaOH or HCl solution. Fermentation was run over a period of 3 days at 30°C temperatures. An irregular result of ethanol yield was obtained at different concentration of particles as shown in Fig 5. Initially from 0 to 5 mg/l, ethanol yield increased from 0.339 to 0.386 g/20 g (0.01695 g to 0.0193 g/g) of biomass. Further increased in particles concentration decreased the ethanol yield. This study demonstrated that Zinc oxide NPs in the concentration rang of 2 to 5 mg/L was capable to enhanced ethanol production by anaerobic yeast fermentation. Beyond 5 mg/l concentration, the NPs showed toxic effect on yeast or any other parameter involved during course of anaerobic fermentation. The highest ethanol

production obtained was 0.386 g/20 g (0.0193g/g) of the plant biomass at 5mg/L of ZnO NPs in contrast to 0 g at 120 mg/L concentration.

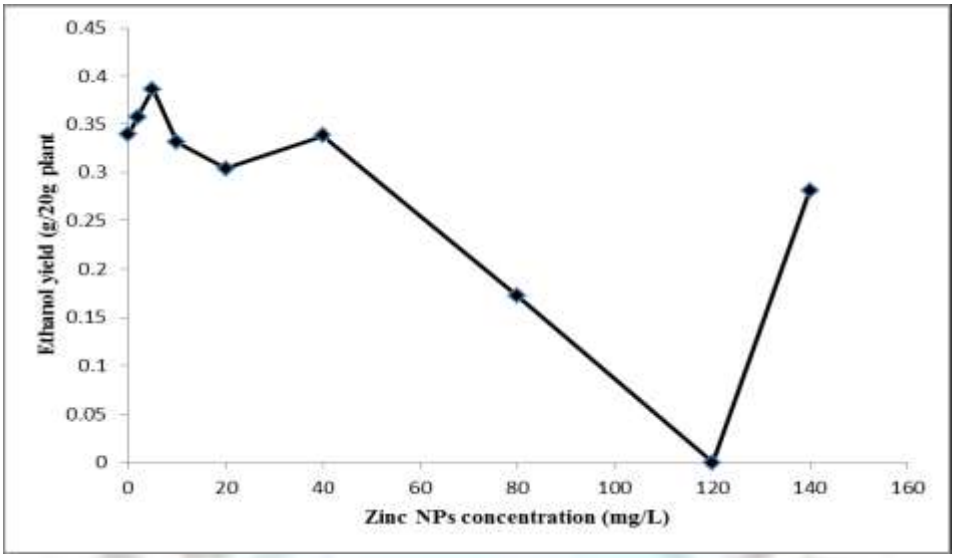

Figure 5: Effect of zinc oxide nanoparticles on ethanol yield.

As can be seen in the Fig 5, no alcohol was produced at 120 mg/L concentration while at 140 mg/L concentration the yield was 0.281 g. Also ethanol yield from 10-80 mg/L was not in sequence. This irregular production of ethanol at different concentration range and no yields at 120 mg/L may be due to contamination in fermentation medium or some other mistake during fermentation process. Ethanol yield under different concentration of zinc NPs was evaluated after each 12hrs of fermentation time. The irregular result obtained is shown in Fig 6. Result showed that at first 12hrs, the yield was higher under all NPs concentration. This means that at first 12hrs about all yeast cells were active, after this the cells activities hindered due to toxicity of zinc oxide NPs. The Fig 6 shows that the highest yield (0.386 g/20 g plant's biomass) obtained at 48hrs of fermentation time under 5 mg/L particles concentration while the least ethanol yield (0.008 g/20 g biomass) was obtained at 60hrs under 120 mg/L of particles concentration. Result also indicated that ethanol yield increases with increasing fermentation time at 0, 2 and 5 mg/L concentration of zinc NPs while at concentration higher than 5 mg/l, the yield decreased with passage of time.

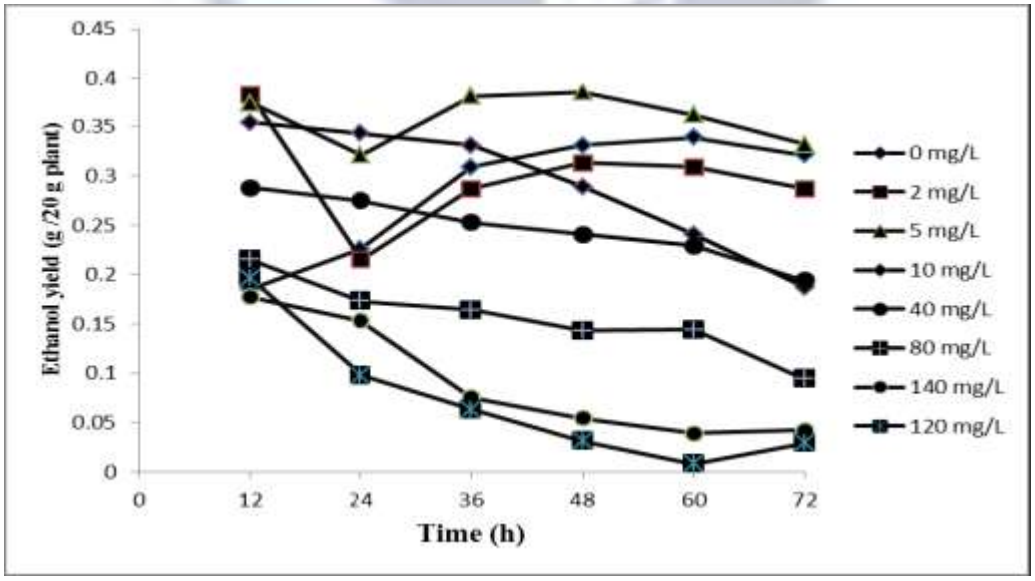

Figure 6: Effect of incubation time on ethanol yield at different concentrations of Zinc oxide NPs.

DISCUSSION

Water hyacinth was used as a source of biofuels like for bioethanol and bio-hydrogen. Water hyacinth contains lignocellulose materials which are composed of mixture of carbohydrate polymers (cellulose and hemicellulose) and lignin. Lignin tightly bound around carbohydrate polymers through hydrogen and covalent bonding. Cellulose part of carbohydrate polymers is a linear carbohydrate consists of glucose molecules which are linked together through 1, 4-glycosidic linkage while hemicellulose is heterogeneous polymers of hexoses, pentoses and sugar acids. Lignin consists of phenyl propane unit which are aromatic polymer and is very complex molecule. The plant biomass is considered to be a potential source of cellulose and hemicellulose for bio-conversion into useful products like bio-hydrogen and bio-ethanol [1, 53].

In present investigation composition of carbohydrates constituents in water hyacinth biomass is hemicellulose 41.6 ± 0.032, cellulose 19.2 ± 0.003 and lignin 4.0 ± 0.003 which show good agreement with the data reported by publishers [1, 54]. Cellulose contents of plant in present study were lower than hemicellulose. It has also been found like this that in plant hemicellulose contents are more than cellulose [2]. It has been reported that plant possess 55% hemicellulose of the total solids present in plant [1]. There are several other studies reported on chemical composition of plant. The difference in plant composition in present and previous studies may be due to different sources or different growth state of plant [4].

Ethanol production from cellulosic materials is carried out by two steps process, saccharification and fermentation. Enzymes are expensive and take several days to completely hydrolyze cellulose into fermentable sugars so it is better to hydrolyze water hyacinth biomass by 1% sulfuric acid solution, which is cheap and consumed less time. However, saccharification by H_2SO_4 produce toxic compounds which may adversely affect rate of fermentation and also it is risky as it is a strong acid, corrosive for skin and causes environmental pollution. In this study dry weight based plant materials produced maximum of 5.0% glucose and 11.02% xylose by dilute sulfuric acid hydrolysis. Products of dry weight based plant by enzymatic hydrolysis were 8.0% and 9.0% glucose and xylose respectively. The main products of cellulose hydrolysis are glucose while that of hemicellulose are xylose.

On complete hydrolysis of cellulose into glucose with a H_2O molecule, the weight ratio of glucose to cellulose come to be: 180/162 = 1.111, while hemicellulose on complete hydrolysis into xylose with a H_2O molecule, the weight ratio of xylose to hemicellulose is: 150/132 = 1.136. Thus, the maximum theoretical glucose derived from 19.2% cellulose in plant biomass is determined as 19.2 × 1.111 = 21.33%; similarly the maximum theoretical xylose derived from 41.6% hemicellulose in plant dry weight based biomass is determined as 41.6 × 1.136 = 47.25%. Total theoretical weight ratio of fermentable reducing sugars (glucose and xylose) derived from dry weight based plant biomass is determined as 21.33 + 47.25 = 68.58%. Present study indicated that 37.50% of total cellulose and 23.70% of total hemicellulose are hydrolyzed into glucose and xylose respectively. Hydrolysis of cellulose and hemicellulose into reducing sugars are low in present study as compared to other studies. Cellobiose is one of the sugars produced in small amount during hydrolysis which is a stronger inhibitor for cellulose hydrolysis and exert its inhibitory effects mainly in initial stage of hydrolysis [55]. Mild conditions and dilute acid may be the reason of low hydrolysis of plant biomass. Reducing sugars production by dilute acid at mild conditions was low as compared to high temperature and concentrated acid [3, 56]. In present study plant biomass is pretreated with only NaOH solution before hydrolysis. However, hydrolysis of biomass can be increased upon with pretreatment with NaOH and H_2O_2. Pretreatment of rice straw with 1% NaOH and H_2O_2 solution at 60°C for 5hrs increased the extent of enzymatic hydrolysis by 53.2% [57]. Also there was an increased in total sugars production using enzymatic hydrolysis when rice hull was pretreated with alkaline hydrogen peroxide [58].

Water hyacinth is the best biomass that can be utilized to produce hydrogen. Literature has reported that the plant is a source for cogeneration of hydrogen and methane [15]. The production of hydrogen in dark fermentation under anaerobic conditions is carried out by using certain species of bacteria such as clostridium with cellulose, starch, glucose and sucrose as substrates [59, 60]. The reaction mechanism of hydrogen generation from glucose and xylose reducing sugars derived from hydrolysis of cellulose and hemicellulose of the plant biomass by hydrogen fermenting bacteria is proposed to be following.

Glucose: $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$
Xylose: $C_5H_{10}O_5 + H_2O \rightarrow 2CH_3COOH + CO_2 + 2H_2$

Above stoichiometric equation shows that 1 mol of glucose can produce 4 mol of hydrogen or 1 g of glucose (molecular weight, 180 g/mol) can produce 498 mL of hydrogen. Therefore, 0.240 g glucose derived from 3 g plant can theoretically generate maximum of 119.52 mL of hydrogen. Second equation shows that 1 mol of xylose can generate 2 mol of hydrogen or 1 g of xylose (molecular weight, 150 g/mol) can produce 298 ml H2. Therefore, 0.27 g xylose derived from 3 g plant biomass can theoretically generate maximum of 80.46 ml H_2. To add up hydrogen generation of both glucose and xylose, 3

g of plant can theoretically produce maximum of 199.98 mL hydrogen. The maximum theoretical yield of hydrogen in dark fermentation using glucose as substrate is 4 mol H_2/mol glucose with acetic acid only byproduct [61]. In present study maximum hydrogen yield is 57 mL/ g of plant (Dry Weight based) which is 85.50% of theoretical maximum hydrogen yield. This study obtained higher hydrogen yield. High yield is due to use of iron nanoparticles in fermentation. Dark-fermentation used was very effective for hydrogen production. Combine dark and photo-fermentation has been used for hydrogen production from the plant biomass and maximum production was 59.6% of theoretical hydrogen yields [62]. Absence of methane in biogas was due to lack methanogens, (methane synthetic microbes) in mixed culture due to pretreatment of culture at high temperature before using as inoculum. High temperature inhibits bioactivity of methanogens. Sucrose and sweet potato has been used as substrate for hydrogen production and maximum 7.1 mole of hydrogen was produced per mole of hexose [63, 64]. However, potato and sucrose are very expensive and water hyacinth biomass is more suitable for it on industrial scale. Production of bio-hydrogen by fermentation in presence of iron nanoparticles is cost effective method as compared to other methods.

Hydrogen production was studied at different concentration of ZnO NPs and result showed that hydrogen yield decreased with ZnO NPs. Presence of heavy metal ions like Zn is known to be fundamental for numerous reactions during anaerobic hydrogen fermentation. However, high concentration of this metal and small size (Nano scale) could inhibit biological hydrogen production in anaerobic fermentation process. Main problem with zinc is that it is not biodegradable and is known to accumulate, reaching toxic concentration [65]. Toxicity of Zn NPs to anaerobic bacteria can be due to dissolved bioavailable Zn ions. Aruoja et al [66] has reported that toxicity of metal oxide NPs is due to higher solubility of NPs in fermentation medium. It is suggested that the toxic effect of micro and nano-sized particles to anaerobic bacteria may be due to different surface area and surface characteristics [29].

Cellulosic biomass is the best feed stock for ethanol production because it is renewable and available throughout earth in large quantities. Selection of cheap and carbohydrate rich raw materials such as weed lignocellulose biomass which contain cellulose (20–50%), hemicellulose (20–35%), and polyphenolica lignin (10–35%) is alternate feed stock for bioethanol production [17, 18, 19, 20]. Yeast enzymes broken cellulose into glucose units and convert it into ethanol. Hemicellulose after hydrolysis into reducing sugars is converted into ethanol and acetic acid by yeast enzymes. Byproducts like glycerol and acetic acid are also possible in fermentation [67]. GC-MS analysis of products produced by common yeast (S. cerevisiae) in fermentation under influence of zinc oxide NPs were ethanol and acetic acid, indicating that common yeasts are able to produce sufficient amount of ethanol. General mechanism of ethanol production from glucose is expressed in following chemical equation.

$$\mathbf{C_6H_{12}O_6 + Yeast\ enzymes \rightarrow 2C_2H_5OH + 2CO_2}$$

Above stoichiometric equation shows that 1 mol of glucose can produce 2 mol of ethanol or 1 g of glucose (molecular weight, 180 g/mol) can produce 0.51 g of ethanol. Therefore, 1 g of glucose obtained from 20 g of plant biomass can theoretically produce 0.51 g of ethanol. These studies focused on bioethanol production by fermentation of plant biomass, the maximum yield was 25-70 mg/g dry mass of plant [2, 3, 56, 68]. Biomass of water hyacinth proved to be a suitable substrate for production of bioethanol [7]. There are several reports on bioethanol production from plant biomass [2].

Ethanol production also increased with zinc oxideNPs and may be explained that NPs stimulates binding of acetaldehyde to alcohol dehydrogenase and its reduction to ethanol. It has been pointed out that presence of Zinc(II) ions in fermentation medium enhance growth rate of yeast cells as well as ethanol production [69]. It has also been documented that the ions promote synthesis of riboflavin, which is essential for microbial cell growth [70]. Genetic level has given a good perception that metal ions play an important metabolic and structural role [71]. The structural role is relevant to hydrogen bonding in DNA structure and plasma membrane. Zinc is an important cofactor for certain enzyme like Zn-superoxide dismutase, responsible for detoxification of yeast cell.

Result showed that high concentration of ZnO NPs in fermentation medium was toxic. Exact mechanism of NPs toxicity to yeast is not known, however, zinc affects permeability of membranes to potassium which causes decrease in yeast growth as well as fermentation activities [72]. Toxicity of ZnO NPs to S. cerevisiae may be due to solubilized Zn-ions. Solubility of metal oxides is a function of concentration [23] and that is the reason why ZnO NPs at higher concentration was toxic to yeast and ethanol yield was decreased. Xia et al [44] also confirmed that dissolution plays an important role in ZnO-induced cytotoxicity when he studied toxicity of Zinc oxide NPs in mammalian cells and showed that ZnO NPs disrupt cellular homoeostasis which leading to cell death by mitochondrial and lysosomal damage. It has been demonstrated by Kovacevic et al [73] that when ZnO concentration increased above 10 mg/L then metabolic process of yeast influenced and respiration activity slows down. In this study when NPs concentration increased above 5 mg/l ethanol production was decreased.

Separation of ethanol from other fermentative products by distillation is a very useful technique. However, water content present in alcohol must be reduced to less than 1% by volume which is difficult by distillation process. Production of bioethanol by fermentation process under the utilization of NPs is cost effective as compared to other process as reported by Nag [74] and others.

Objective of present study was to investigate enhancive effect of zinc oxide nanoparticles on fermentative bio-hydrogen and bioethanol production from water hyacinth biomass. This study may support researchers in seeking a better understanding of biofuel production from biomass by using nanotechnology. It may help to generate knowledge base that will significantly improve biofuels synthesis.

Conclusions

This study demonstrates that fermentative production of ethanol and hydrogen from water hyacinth is a commercially potential and sustainable process. Zinc oxide nanoparticles significantly affect hydrogen and ethanol production. Ethanol production is enhance by zinc nanoparticles.

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