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Effect of Iron Nanoparticles on Hyacinth's Fermentation

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Abstract: Biomass feedstock is desirable for bio-hydrogen and bioethanol production as they have less competition with food crops and are hard to be localized geographically. Water hyacinth (*Eichhornia crassipes*) is the fastest growing plant, containing abundant of cellulose and hemicellulose which can be easily converted into fermentable sugars and is more suitable feedstock for bio-hydrogen and bioethanol. In this study bio-hydrogen and bioethanol were produced from dry biomass of water hyacinth by microbial fermentation under influence of iron nanoparticles. For fermentative bio-hydrogen production dry powdered 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 with 1% sulfuric acid solution, autoclaved at 121°C, 15 lbs for 1.5h. The reducing sugar obtained in this method containing 5% glucose. Results showed that the specific concentration of iron nanoparticles was able to enhance the hydrogen yield. Ethanol yield was enhanced by iron nanoparticles by using it in certain concentration range during fermentation. Maximum hydrogen yield of 57mL/g of dry weight based plant biomass was obtained at 250mg/L concentration of iron nanoparticles which is 85.50% of the maximum theoretical yield. The maximum ethanol yield of 0.0232g of dry weight plant biomass was obtained at concentration of 150mg/L of iron nanoparticles. The ethanol yield constitutes 90.98% of the maximum theoretical yield at iron nanoparticles.

Keywords: iron nanoparticles, water hyacinth, yeast *Saccharomyces cerevisiae*, fermentation, biohydrogen, bioethanol

Introduction:

Due to rapid increase in the world's population and developing technologies, need for energy is increasing day by day. The current fossil energy resources (oil, gas and coal) which are the main fuel of our industrial economies and consumer societies, with limited reserves are decreasing [1]. In addition, these energy resources are also responsible for the adverse effect of greenhouse gas emission on environment. The development of technologies for generation of new and renewable source of energy like biomass, solar power, geothermal, wind and hydropower is the need of the time [2]. 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 [3, 4]. 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 [3, 5, 6, 7, 8, 9, 10].

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 [6] as it has significant amount of hemicellulose and very less amount of lignin content and is a suitable biomass for bioethanol production [11]. The production of bio-gases (bio-hydrogen, bio-methane etc.), increase with addition of nickel to plant or mixture of plant and cow dung during fermentation [12]. Plant collected from polluted water contains sufficient amount of heavy metals. The addition of mentioned metals in nano scale range may increase production of biofuels by fermentation.

The process of fermentation is complicated and is affected by many factors 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,

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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 [13]. The most important parameters affecting the process of hydrogen fermentation are four, pH, temperature, organic loading concentration and hydraulic retention time [14]. 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 [15].

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 [16]. Hydrogen consuming bacteria from hydrogen reactor can be avoided by the heat-treatment of seed sludge [17], low pH operation [18] and by addition of inhibitors [19]. 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 [20]. 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 [21, 22, 23, 24].

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 [25].

Iron nanoparticles (NPs) are one of the most studied nanomaterials. They have been used in many fields of study such as medicines, cell biology, environmental technology, mining, diagnostic and analytical chemistry [26]. They have novel properties especially high surface to volume ratio and inherent biocompatibility [27] and are used in biomedical and environmental applications like delivery of drugs, pollution remediation, destruction of tumor tissues and magnetic bio-separation [28, 29, 30]. They are also used in magnetic drug targeting systems [31] and contrast agents in magnetic resonance imaging [32]. 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 [33]. In these elements, iron is the most important trace element requires for hydrogenase and other enzymes which are involved in bio-hydrogen production [34]. On the active site of hydrogenase enzyme iron is bonded and catalyzed the reduction of proton to hydrogen [35]. The effect of iron on fermentative hydrogen production is documented in both batch [36, 37] and continues culture [36, 38]. The fermentative hydrogen production by using anaerobic mixed culture, increase with utilization of iron. Iron NPs modify metabolic pathway of microorganisms. The effect of gold NPs on production of fermentative hydrogen is reported and found that hydrogen production increases with decreasing the size of gold NPs [39].

Some species of bacteria have no nutritional requirement for iron [40, 41] but those bacteria which are involved in fermentation of hydrogen i.e. *Clostridium* are dependent on iron. Also some enzymes in yeast are dependent on iron.
There are no adverse effects of iron NPs on microorganisms. As an example *E. coli* were grown in a medium containing iron NPs. The results showed that no adverse effect was found on growth of these organisms, but high concentration of iron can inhibit activities of hydrogen producing bacteria and ethanol producing yeasts [39, 42].

Main aims and objectives of this study were 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 iron nanoparticle's (oxide form) 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 were used without any further purification. Majority of the chemicals were purchased from Merck Germany/Sigma Aldrich/BDH and Fluka. For

culture media preparation and microbiological studies, most chemicals and media compounds including yeast extract, peptone, and tryptone were obtained from BDH Laboratory Chemical Division (Pool, Dorset, England) and DIFCO Laboratory (Detroit Michigan USA), Merck (Darmstadt, Germany). Deionized water was used for preparation of various solutions.

Water hyacinth fresh plant were collected from a natural pond in Taxila, situated at 33°45'0"N 72°48'36"E, the North-Western edge of the Punjab province of Pakistan [43]. The plant was washed several times with tap water to remove adhering dirt and soil. It was chopped into small pieces and dried at 105°C in a hot-air oven for 12hours. A part of the dry material was stored in plastic bags at room temperature while the other part was milled and the powdered material was passed through 0.8 mm pore sized mesh sieve for use [43]. 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. Then the samples were washed with tape water using a 38^{-1}mm 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 250mL flasks. Five grams of pretreated plant materials was taken in each flask and were autoclaved at 121°C for 20 minutes. Then 50mL 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 24 hours with rotation at rate of 150rpm. Saccharification efficiency was calculated as percentage conversion of pretreated biomass to reducing sugars.

Acid hydrolysis of water hyacinth was carried without pretreatment. 50mL of 1% sulfuric acid solution was taken in 100mL flask and 3 g of the plant powder was added to it. Flask was autoclaved at 121°C, 15 lbs for 1.5h. 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 5h. 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. 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 10h and stored at 4°C as a stock culture. *Clostridium butyricum* was activated by mixing 1mL of stock culture with 10mL of fresh tryptone sucrose yeast (TSY) extract medium

in serum bottle. TSY used, contained 5.0g tryptone; 3.0 g sucrose; 5.0g yeast extract and 1.0g K_2HPO_4 per liter. Argon gas was flushed into serum bottle to ensure anaerobic condition. Medium was incubated at 37°C for 12h at 150rpm 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 7540mg, K_2HPO_4 250mg, Na_2CO_3 4000mg; $CuSO_4.5H_2O$ 10mg; $MgCl_2.6H_2O$ 200mg, MnSO4.4H2O 30mg, $FeSO_4.7H_2O$ 50mg, NaCl 0.1g, $CaCl_2$ 0.01g, $NaMoO_4.2H_2O$ 0.01g, $Na_2S.9H_2O$ 0.25g, $CoCl_2.6H_2O$ 0.250mg and yeast extract 2.0g. Medium was autoclaved for removal of contamination before using in fermentation.

Dark-fermentation for hydrogen production was performed in 120mL serum bottles in batch tests. Into each serum bottle, 40mL of inoculum, 40mL of autoclaved nutrition medium and enzymatically hydrolyzed plant hydrolysate obtained from 3g of plant biomass was added. Total fermentation liquor volume of each bottle was adjusted to 100mL 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 60mL syringes were inserted to collect total biogas and placed in reciprocal shaker. Iron nanoparticles were used in concentration range of 0-500mg/L in fermentation medium to check its effects on hydrogen production efficiency while concentration of plant hydrolysate was kept fixed. Zero mg/L concentration of nanoparticles was taken as control. 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 250mL 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 24hours at about 150rpm shaking. After 24h incubation period, flasks were removed from shaker and inoculum was used for fermentation medium.

Table 1: Inoculation medium

Compound	**Used amount/Concentration**
Glucose	1g / (20g/L)
Peptone	1g / (20g/L)
Yeast extract	0.5g / (10g/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 250mL Erlenmeyer flasks with working volume of 100mL. Fermentation medium in each flask contained concentrated hydrolysate of 20g powdered plant biomass, 4.0mL supplementary nutrition solution (composition mentioned in Table 2), 2.0mL yeast inoculum and iron nanoparticles with concentration range from 0 to 600mg/L. Control medium (without NPs) was prepared.

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 4.0mL 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 200rpm. All experiments were carried out in triplicate. Lignin, cellulose and hemicellulose contents were determined by detergent extraction method [44].

Composition of water hyacinth hydrolysate for total producing sugars, from both enzymatic and acid hydrolysis, was measured by DNS (3, 5-dinitrosalicylic acid) method [45]. A UV/VIS-scanning spectrometer of double beam was used for measuring absorbance. For this purpose 2.0mL of DNS reagent was taken in a test tube and 1.0mL hydrolysate was added to it. Blank containing 2.0 ml of DNS and 1.0mL 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. 1.0mL of potassium sodium tartrate solution was added to mixture in order to stabilize color. After this, test tube was cooled at room temperature; added 8mL of distilled water and the absorbance was measured with a spectrometer at 540nm. The amount of reducing sugar was calculated by adopting the following formula.

$$\text{RSY (\%)} = \frac{\text{(Reducing Sugars Concentrations mg/mL) 50mL X 100\%}}{\text{(Substrate Added, mg)}}$$

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 2m 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 12mL per minute. For GC analysis, gas samples were collected through a hypodermic needle. A gas sample of 5mL 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 200-250µl 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: 7.5m, film: 20µm). Program conditions were as such, temperature program: 125°C, column temperature 125°C, detector temperature 250°C, injector temp 250°C, linear velocity 200cm/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 0.99715g/cm^3). Reading of each sample was taken at 20°C.

Analytical grade reagents, ferric chloride hexahydrate, sodium hydroxide and distilled water were used for synthesis of iron nanoparticles. 1.0M iron chloride hexahydrate ($FeCl_3.6H_2O$) solution was prepared with working volume of 50 ml and 6.0M NaOH solution was added to it drop wise with constant stirring until pH was reached to 7. A precipitate of $Fe(OH)_3$ was formed and trace of Fe(II) was transferred to it. Final pH of $Fe(OH)_3$ was readjusted to 7 with dilute solution of NaOH. Volume of solution was adjusted to 100mL and was stirred with magnetic stirrer on hot plate. Then under vigorous stirring mixture was heated to boiling and refluxed for certain time. Finally it was filtered off, washed with distilled water and dry iron nanoparticles in oxide form were obtained (Hematite/Fe_2O_3). The mixture was transferred to refluxing pot after complete dissolution and was refluxed at 65°C for 6h. After refluxing mixture for

6h white colored precipitate was appear 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 1h in air and nanoparticles were obtained.

Two experimental techniques, X-ray diffraction (XRD) [4, 46] and Scanning Electron Microscopy (SEM) were used for characterization of iron nanoparticles. Phase identification of iron nanoparticles was carried out by X-ray diffraction on a Bruker D8 ADVANCE diffractometer with CuKα radiation (λ = 0.15418nm). 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 [43].

Dilute sulfuric acid hydrolysis under reflux at 110°C for 3h was very effective in releasing good amount of fermentable sugars than autoclaving at 121°C for 1.5h. 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 1.5h autoclaving using 1% sulfuric acid solution was 2.5 g/50 g (0.05g/g) of dry weight (DW) plant biomass. After 5h 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 1.0g, D-galactose 0.8 g, L-arabinose 1.1g, D-mannose 2.0g and D-xylose was 5.6g. 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. 3: 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					

Results:

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. 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 8.5g/50g (0.17g/g) of dry weight plant's biomass after reaction of commercial enzymes for 24h. Out of 8.5g of concentration of glucose was 4.0g and that of xylose was 4.5g. 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 4: 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

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 [47]. 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) pattern of iron NPs was obtained with Cu Kα radiation (λ = 1.5418 A°) and result is illustrated in the Fig. 1. All peaks in the Fig 3 belong to hematite phase according to JCPDS card No 33-0664. Major XRD peaks were obtained at angle (2θ) 23.17°, 33.39°, 35.89°, 50.77°, 54.29°, 62.74° and 63.96° corresponding to indices (012), (104), (110), (024), (116), (214) and (300) respectively. From afore-mentioned indices of XRD pattern it is cleared that nanoparticles of iron prepared were pure. Peaks positions and their intensities were compared with Joint Committee for Powder Diffraction Standards (JCPDS) card no 33-0664 for examining phase structure and purity. XRD pattern indicated that all diffraction peaks were readily indexed to rhombohedral phase α-Fe_2O_3 without presence of any impurity. Average particle diameter D was obtained from main peaks by using Scherer's formula, D = 0.9 λ/ (β cos θ), here λ is the wavelength (Cu Kα), 0.9 is the machine constant, β is the full width at the half-maximum (FWHM) and θ is Bragg diffraction angle or peak position. Using above formula average size of iron NPs was 11.59nm.

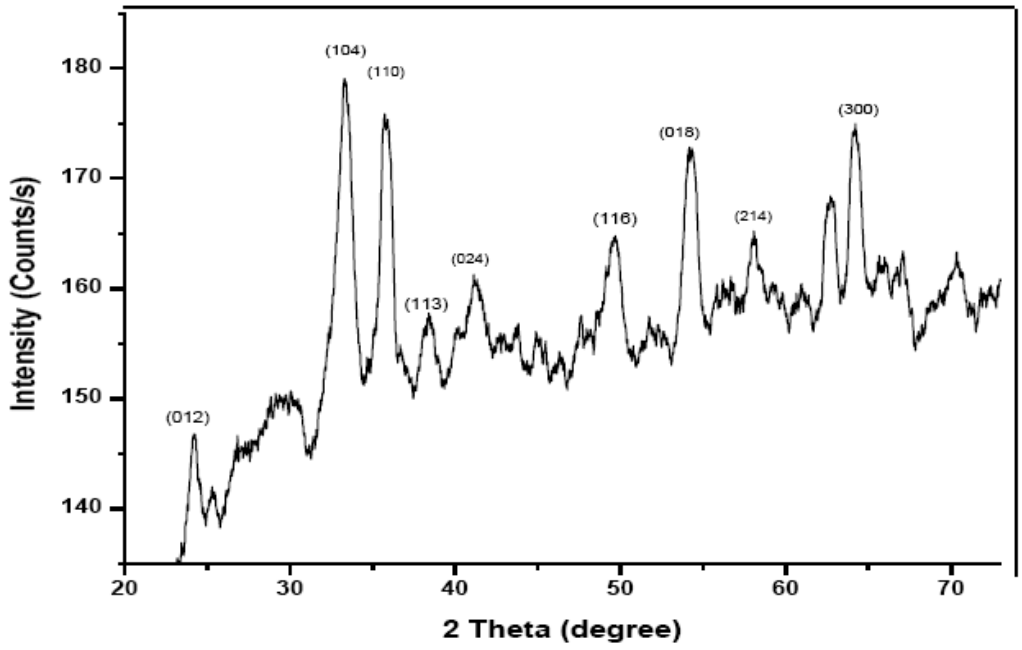

Figure 1: XRD pattern of iron nanoparticles

Table 5: Calculating particle crystalline size of Iron NPs from FWHM of X-ray diffraction pattern

Phase	Peak position	FWHM (in radian)	Cos θ	Particle size (nm)
104	33.39	0.010304	0.957873	12.95
110	35.89	0.006182	0.951353	21.44
116	54.29	0.012365	0.889855	10.02
300	63.96	0.060314	0.848233	01.95

Scanning electron microscopy (SEM) was used to investigate surface morphology of iron NPs. Fig. 2 shows SEM image of freshly prepared particles. Morphological information obtained from SEM analysis was very qualitative and much more reliable in identifying the structure, showing that nanoparticles are in form of nanospheres.

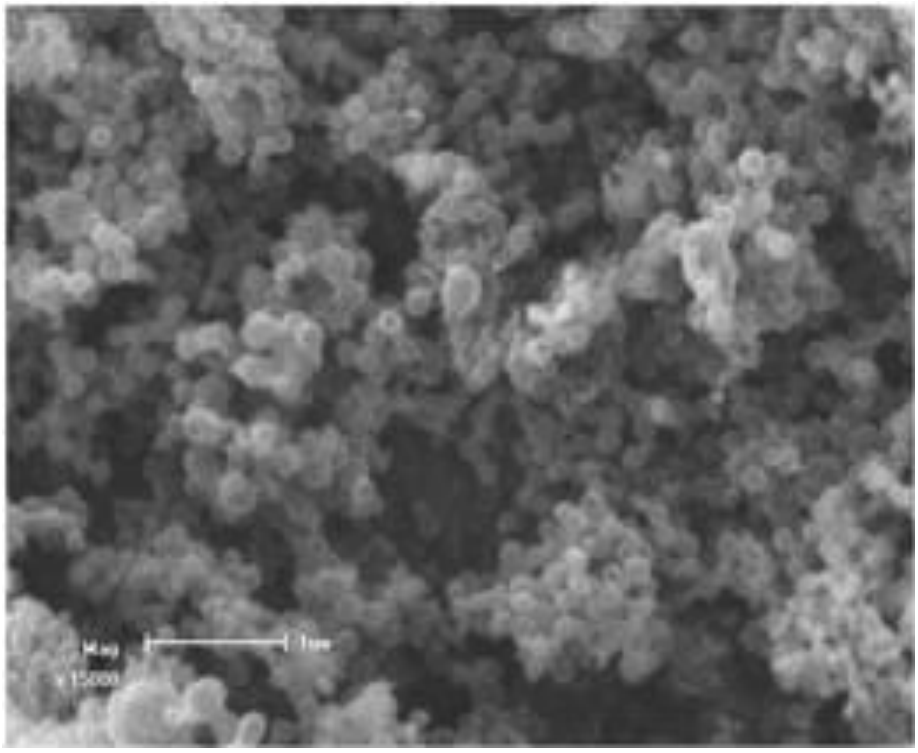

Figure 2: SEM image of iron nanoparticles

Effect of iron NPs on hydrogen production was investigated by testing hydrogen yield at different concentration of iron NPs in batch experimental models. Initial pH of fermentation media were adjusted at 7.0. The final pH of each batch experiment was checked which was decreasing as the concentration of NPs increased. The plant hydrolysate used as substrate for hydrogen fermentation contained glucose and xylose reducing sugars in concentration of 8.0% and 9.0% respectively. Batch experiments were carried out individually and each experiment continued until evolution of hydrogen from each serum bottle stopped as well as no quantity of reducing sugar was found in metabolites. Anaerobic fermentation of plant hydrolysate produced hydrogen and carbon dioxide as dominant biogases. Methane and hydrogen sulfide were not produced, indicating methanogen and sulfate producing bacteria were absent in fermentation medium. During the course of anaerobic hydrogen fermentation, concentration of iron NPs used was in ranged of 0 to 500mg/L. The effect of various concentrations of particles on hydrogen production at initial pH of 7.0 during fermentation is illustrated in Fig. 5. Maximum hydrogen yield was 171mL/3g (57mL/g) plant (dry weight based) when the concentration of particles was 250mg/L, but when particles concentration was higher than 250mg/L the slope of the curve decreased.

This study showed that hydrogen yield during was fermentation increased by increasing concentration of iron NPs from 0 - 250mg/L, however, when concentration of particles increased than 250mg/L, the hydrogen quantity tend to decreased. This demonstrated that in certain concentration range iron NPs were able to enhance hydrogen production. While at much higher or lower concentration than suitable, was not conductive for raising hydrogen production.

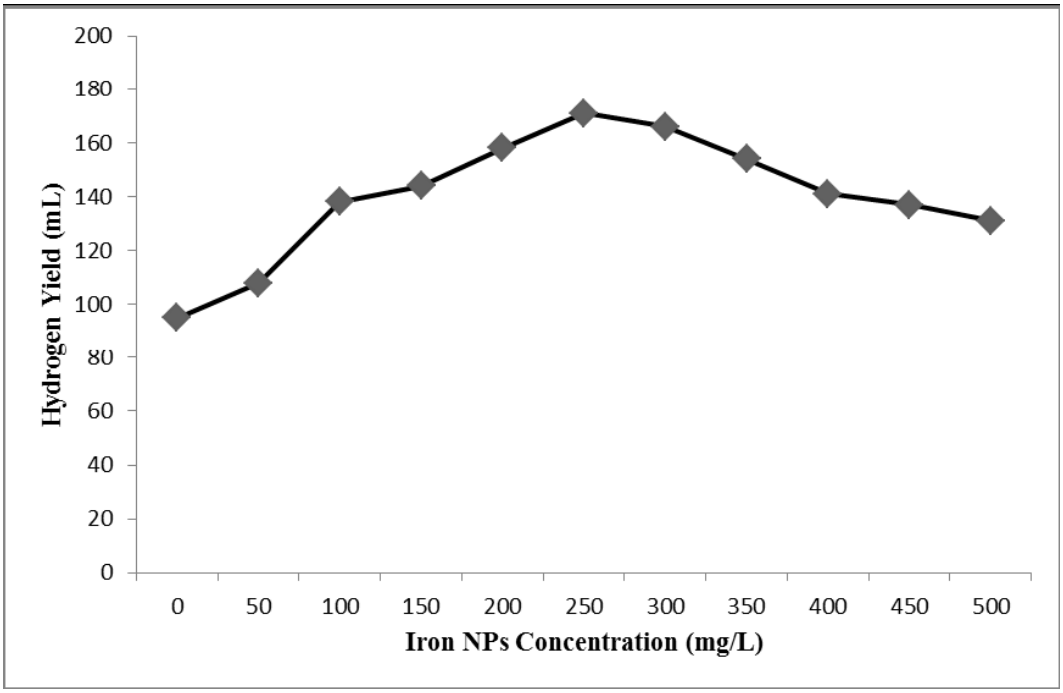

Figure 3: Effect of iron nanoparticles on hydrogen yield

Fig. 3 shows that hydrogen yield even in presence of 500mg/L NPs, exceed than control from 95mL to 131mL. It showed that iron NPs are not harmful for the microbes involved in hydrogen fermentation and promotes the hydrogen production of plant biomass. Hydrogen yield was defined as the total hydrogen produced by hydrolysate of 3g of plant biomass. The maximum hydrogen production at 250mg/L of iron NPs was 55% higher than control experiment.

Final pH in all batch fermentation was checked at the end of experiments and was lower than the initial pH 7.0 due to addition of iron NPs. The final pH of control test was 6.8 and tended to decrease with increasing concentration of particles. At lower iron NPs concentration the decrease in pH was little; however when the concentration of particles was increased from 350mg/L the decrease in pH was much higher. Lowest final pH of 6.1 was obtained at 500mg/L, the highest concentration of particles used in this study. The final pH at 250mg/L iron NPs, concentration at which maximum hydrogen was produced, was 6.5. This is suitable pH for hydrogen production by anaerobic mixed culture. Production of hydrogen in fermentation process is dependent on pH however, pH in serum bottles were ranged from 6.1 to 6.8 during incubation for all tested NPs. Since most of microbial species carried hydrogen fermentation such as *Clostridium* functions within a pH range of 6.0-6.8, pH did not adversely affect the microbial activity.

The liquid part of fermentation was tested for the soluble metabolites produced by mixed culture. The metabolites produced during fermentation were acetic acid and very small amount butyric acid and ethanol. However their production under the effect of various iron NPs concentration was not studied.

Fig. 4 illustrates effect of fermentation time on hydrogen yield under 250mg/L iron NPs concentration. Results showed that anaerobic fermentative hydrogen production finished within 48 h. Average rate of hydrogen yield in first 24h was 2.0mL/h. From 24 to 36h, the rate was 4.5mL/h and from 36 to 48h the rate was 5.5mL/h. After 48 h there was no increased in the rate of hydrogen yield. The result suggested that in presence of 250mg/L iron NPs fermentation completed in 48h; mean NPs decreased lag time of hydrogen production.

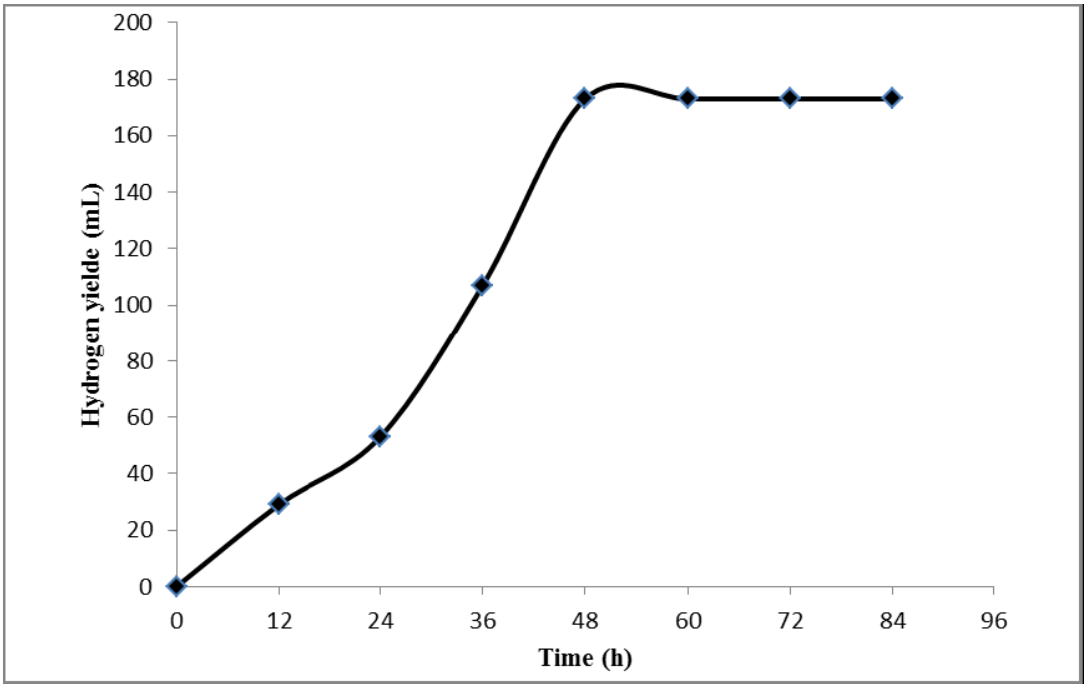

Figure 4: Effect of incubation time on hydrogen yield at 250 mg/L concentration of iron NPs.

The metabolites such as acetic acid, ethanol etc. may be produced during fermentation but their analysis was monitored in this study.

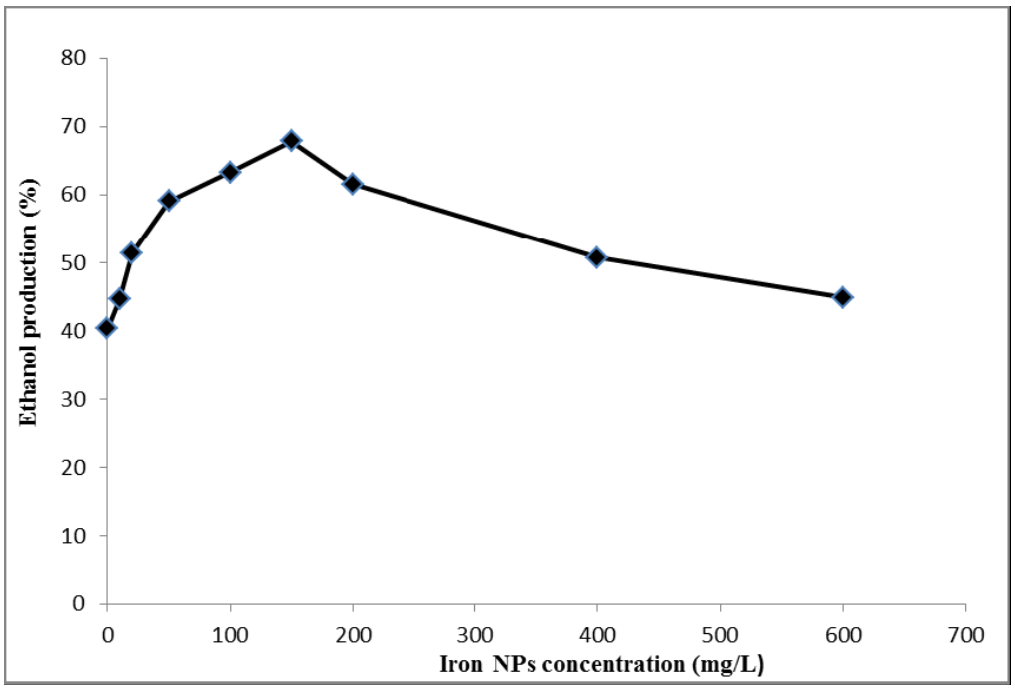

Figure 5: Effect of iron nanoparticles on ethanol yield

Effect of Iron NPs on ethanol fermentation by yeast, *Saccharomyces cervicea* was investigated. The chloroform based extract of anaerobic fermentation of water hyacinth hydrolysate by yeast was analyzed with GC-MS. Results showed ethanol (C_2H_5OH) and acetic acid (CH3COOH) as dominant products. Ethanol yield (g/20g plant dry weight) enhanced with addition of iron NPs. The NPs was added at concentration range of 0, 10, 20, 50, 100, 150, 200, 400, and 600mg/L. Acid autoclaved hydrolysate obtained from 20g of dry powdered plant materials was used as source of glucose for ethanol production. Initial pH was adjusted at 4.5 with NaOH and HCl solutions as mentioned in materials and method. Fermentations were run over a period of 72h.

Results demonstrated that ethanol production in presence of iron NPs increased to a maximum of 0.464g/20g (0.0232g/g) plant biomass. Fig. 5 show that increase in NPs concentration from 0 to 150mg/L, increased production of ethanol from 0.339g to 0.464 g/20g of plant biomass. Further increase in particles concentration decreased ethanol production. So the highest ethanol production was obtained at 150mg/L of iron NPs concentration. The result also showed that ethanol production at 600mg/L which was the highest concentration of particles used in this study was higher than control (0mg/L). It suggested that Fe NPs are not harmful for anaerobic fermentative ethanol production by *Saccharomyces cerevisiae*.

Ethanol yield was checked after every 12h during fermentation under different concentrations of iron NPs. Result obtained is illustrated in Fig. 6. The result showed that rate of ethanol production increased from 12 to 48h of fermentation time and after 48 to 72h the rate decreased with the increased in time. The highest ethanol yield was 0.459 g/20g plant's biomass after 48 h of incubation time at 200mg/L concentration of NPs. However a small deviation has occurred in result, as ethanol yield in case of 50mg/L and 100mg/L of particles increased even after 48h. Maximum ethanol yield at 50 and 100 mg/l concentration of NPs were 0.397 and 0.388 g/20g plant biomass's hydrolysate respectively at 60 h of fermentation time.

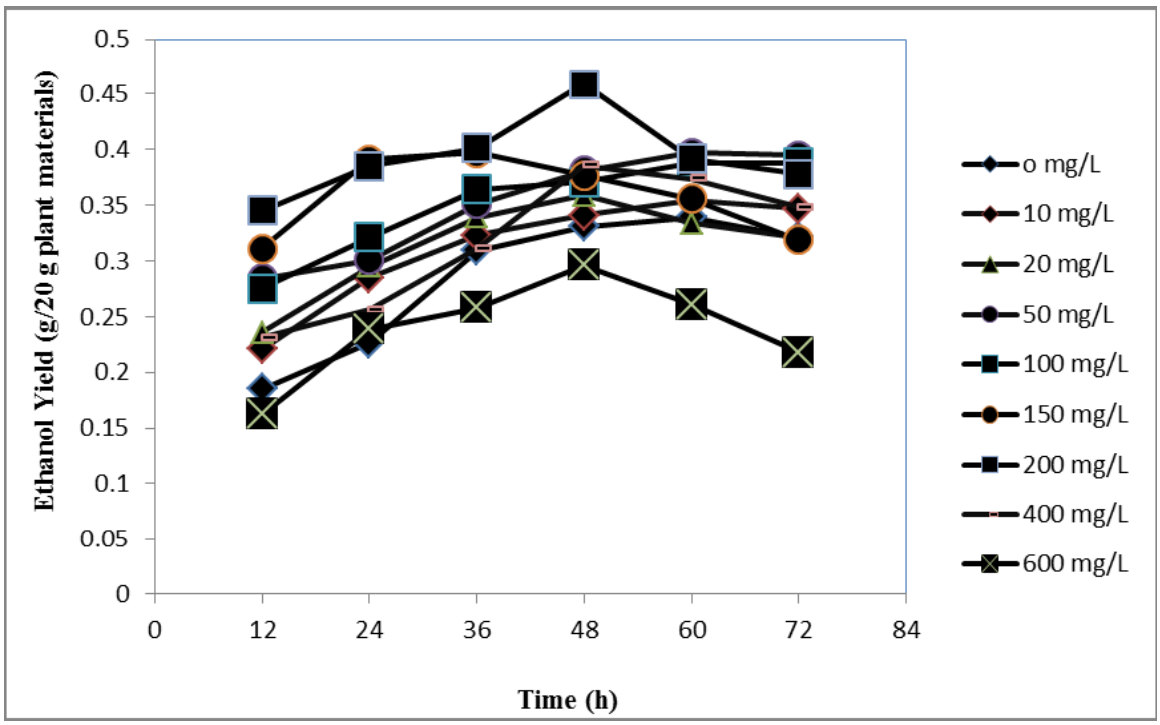

Figure 6: Effect of incubation time on ethanol yield at various concentrations of Iron NPs

As no alcohol was produced at 120mg/L concentration while at 140mg/L concentration the yield was 0.281g. Also ethanol yield from 10-80mg/L was not in sequence. This irregular production of ethanol at different concentration range and no yields at 120mg/L may be due to contamination in fermentation medium or some other mistake during fermentation process.

Discussion:

Most of industrial effluents containing nanoparticles discharge to nearby soil and water bodies, adversely affecting environment, human health and safety. Cleaning of water bodies and damp soil from nanoparticles through conventional methods is expensive. Development of environmental friendly technologies for remediation of soil and waste water polluted with NPs is topic of global interest. Uses of metal accumulating plants such as *Thlaspi caerulescens* in remediation of toxic materials from waste water has been recognized [48]. There are more than 400 hyper accumulator plants. It has been shown that *Thlaspi caerulescens* accumulate 26,000ppm of heavy metals without showing any injury in plant parts [49]. Water hyacinth was used as a source of biofuels like for bioethanol and bio-hydrogen. 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 [50].

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 [5, 51]. 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 [6]. It has been reported that plant possess 55% hemicellulose of the total solids present in plant [5]. The difference in plant composition in present and previous studies may be due to different sources or different growth state of plant [8].

Table: 6 Chemical composition of water hyacinth according to different sources

Parameter (% on DM basis)	This study	[84]	[85]	[86]	[87]	[88]	[89]
Hemicellulose	41.6 ± 0.032	32.69	33.39	43.40	18.42	33.40	22.00
Cellulose	19.2 ± 0.003	19.02	18.00	17.80	25.61	19.50	31.00
Lignin	4.0 ± 0.003	04.37	26.36	07.80	09.93	09.27	07.00

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 [52]. 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 [7, 53]. 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 5 h increased the extent of enzymatic hydrolysis by 53.2% [54]. Also there was an increased in total sugars production using enzymatic hydrolysis when rice hull was pretreated with alkaline hydrogen peroxide [55].

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 [56]. 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 [57, 58]. 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 1g of glucose (molecular weight, 180g/mol) can produce 498 ml of hydrogen. Therefore, 0.240g glucose derived from 3g plant can theoretically generate maximum of 119.52mL of hydrogen. Second equation shows that 1 mol of xylose can generate 2 mol of hydrogen or 1g of xylose (molecular weight, 150g/mol) can produce 298mL H2. Therefore, 0.27g xylose derived from 3g plant biomass can theoretically generate maximum of 80.46mL H_2. To add up hydrogen generation of both glucose and xylose, 3g of plant can theoretically produce maximum of 199.98mL 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 [59]. In present study maximum hydrogen yield is 57mL/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 [60]. 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 [61, 62]. 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.

In this study maximum hydrogen yield, 57mL/g biomass produced at 250mg/L iron NPs

concentration. Red sediment formed in the bottom of serum bottles after the addition of 250mg/L iron NPs to fermentation medium. Similar sedimentation has also been reported by Hongliang *et al.*, [63]. TEM analysis of red sediment has been investigated which has showed that iron NPs stuck to bacterial cells surfaces [63]. It is documented that interaction of iron NPs and bacteria changed the bacterial morphology. Bacterial morphology became longer and thinner in presence of iron NPs. However, there are also several other factors such as culture medium, growth stages of bacteria etc. which affect bacterial morphology, so the cause of change of bacterial morphology is not clear and require further investigation. The immobilization of mixed bacteria cells on iron NPs followed by increased hydrogen production may be a proposed mechanism. This enhanced the chances of bacteria to meet substrate. Bio-hydrogen production improved when bacteria cells was immobilized on $FeCO_3$ sediment [64]. Detection of strong affinity has reported within few hundred nanometer distance between *Shewanella oneidensis* (a metal-reducing bacterium) and goethite (a-FeOOH) under anaerobic condition [65].

Higher hydrogen production in the presence of iron (hematite) NPs may be explained on the fact that iron slowly released from hematite source which kept the proper iron concentration and inhibited high iron concentration which could be toxic for bacteria. Proper iron could promote enzymes (mostly hydrogenase) activities and growth of bacteria, thereby results maximum hydrogen production. It has been reported that hydrogenase activity decreased with deficiency of iron [66]. The mechanism could also be explained on Oxidation Reduction Potential (ORP) value. It has reported that addition of iron NPs to fermentation medium decreased ORP value. Low ORP value was beneficial for hydrogen production of bacteria in anaerobic fermentation process [67]. However, further investigation is needed to explained detailed mechanism of iron NPs on hydrogen production.

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 [21, 22, 23, 24]. 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 [68]. GC-MS analysis of products produced by common yeast (*S. cerevisiae*) in fermentation under influence of iron and zinc 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.

$C_6H_{12}O_6$ + Yeast enzymes → $2C_2H_5OH + 2CO_2$

Above stoichiometric equation shows that 1 mol of glucose can produce 2 mol of ethanol or 1g of glucose (molecular weight, 180g/mol) can produce 0.51g of ethanol. Therefore, 1g of glucose obtained from 20g of plant biomass can theoretically produce 0.51g of ethanol. In present study maximum ethanol yield is 0.0232g/g of plant biomass when 150mg/L iron nanoparticles were used which is 90.98% of maximum theoretical yield. Maximum ethanol yield in the absence of iron NPs is 0.01695 g/g of plant biomass which is 66.47% of maximum theoretical yield. On basis of these experimental results it may be interfered that ethanol production was enhanced with iron NPs. This clearly depicts the positive role of iron NPs in ethanol production. Other studies focused on bioethanol production by fermentation of plant biomass, the maximum yield was 25-70mg/g dry mass of plant [6, 7, 53, 69]. Biomass of water hyacinth proved to be a suitable substrate for production of bioethanol [11]. There are several reports on bioethanol production from plant biomass [6].

As mentioned earlier that ethanol production increased with increasing iron NPs from 0 to 250mg/L and further increased in concentration led to decrease production. Exact mechanism of ethanol production under different concentration of iron NPs was not known and requires further investigation. However, iron is required in nanomolar range as trace element necessary for growth of yeast cells [70]. It could be suggested that iron NPs acts as cofactor in several enzymes in yeast cells. Availability of iron is of greater significance and is limiting factor of yeast growth. Iron is an essential element for growth and metabolism of yeast cell and it directly used for cytochrome synthesis. Iron (Fe_2O_3) NPs is said to be a significant source which released iron in suitable amount for yeast cells in fermentation media.

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 [71] and others.

Objectives of present study were to investigate enhancive effect of iron and zinc 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 nanobiotechnology. 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. Iron nanoparticles significantly affect hydrogen and ethanol production. Iron nanoparticles enhance fermentative hydrogen production. Ethanol production is enhancing by both iron nanoparticles. For fermentative hydrogen production optimum iron nanoparticles concentration is 250mg/L and for ethanol production optimum iron nanoparticles concentrations are 150mg/l. These concentrations are besides that already present in dry biomass of plant. Maximum hydrogen yield is 57mL/g of the plant biomass which is 85.50% of theoretical maximum hydrogen yield. The maximum ethanol yield is 0.0232g/g of the plant biomass which is 90.98% of maximum theoretical yield. This study indicates that water hyacinth accumulate different types of nanoparticles.

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