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ALKALINE PRE-TREATMENT OF WHEAT STRAW FOR PRODUCTION OF ETHANOL USING *SACCHAROMYCES CEREVISIAE* SS-4 IMMOBILIZED IN NANOPARTICLES

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Abstract

This study was designed to evaluate cost effective bioprocess to enhance production of bioethanol by utilizing immobilized *Saccharomyces cerevisiae* SS-4 in calcium alginate (Ca) beads and calcium alginate magnetic nanoparticles (Ca-MNP) beads to improve bio ethanol production using wheat straw. The method of pre-treatment had a pronounced effect on the yield of reducing sugars. Maximum delignification (58%) and 36.31% saccharification was observed with 2.5% (v/v) NaOH. As concentration of NaOH increaseD, cellulose content decreaseD while increase in weight loss was observed. Results showed that immobilized cells of *S. cerevisiae* SS-4 in Ca-MNP gave high yield of ethanol (49.71g/L) compared to immobilized cells of *S. cerevisiae* SS-4 in Ca (45.66 g/L) and free cells (36.52g/L) at pH 4.5, 28 ºC for 72 hrs. Magnet recovery method was used to recover nanoparticles and reusability was evaluated after five time usage.

Key words: Wheat straw, Alkaline pre-treatment, Ethanol, Magnetic nanoparticles, Immobilization, Na alginate, *S. cerevisiae* SS-4, fermentation.

Introduction

The ever increasing exploitation of fossil fuels, unpredictable and increasing petrol prices, climate change and political influences are main factors that paved the way for alternative bioenergy sources for fossil fuels (Davis *et al*., 2005; Cazetta *et al*., 2007). The sustainable solution of this problem is to produce bio-ethanol from biomass which can also replace petroleum products to avoid its high prices in an effective way. This will also reduce the effects of global warming by eliminating its harmful environmental impacts resulted by fossil fuels utilization throughout the world additionally putting less pressure on oil reserves (Saini *et al*., 2015). Ethanol has been produced by various biomass feed stock which contain appreciable sugar content. Sugarcane and corn also have been utilized to produce bioethanol but not preferred because of competition with food industry.

Wang *et al*., (2007) used lignocellulosic biomass to produce bioethanol which had a potential to minimize green house gas emissions up to 86%. This is underutilized biomass as carbon source which is renewable and abundantly available (Qu *et al*., 2006). This can minimize carbon source load on grain-based crops as well as food based crops for production of fuel as biofuel. Lignocellulosic biomass is easily available source of fermentable sugars that can readily be fermented into bioethanol by using enzymes and can substitute gasoline products by producing bioethanol (Dutta *et al*., 2014). Lignocellulosic biomass consists of lignin, hemicelluloses and cellulose (Vallejos *et al*., 2012). According to reports 491 billion litters of bioethanol can be obtained from waste crops and crop residues while 442 billion litters are produces annually by using lignocellulosic biomass. These cellulosic materials are renewable and are available at cheaper rates (Gupta & Verma, 2015). Sugarcane bagasse, rice straw, wheat straw and corn stover are lignocellulosic material that can generate bioethanol productions abundantly and act as reliable source (Li *et al*., 2008).

Chemical composition of wheat straws shows that it contains proteins, carbohydrates (cellulose, lignin and hemi-cellulose), minerals including (calcium and phosphorous), detergent fibers, silica and ash. The straw is also rich in vitamins and other bioactive compounds along with these components (Slavin, 2003). Composition of these micronutrients and macronutrients which vary accordingly from crop to crop (Safdar *et al*., 2009), kind of soil, environmental conditions, fertilizers applied and stage of plant growth (Yasin *et al*., 2010). Wheat straw is considered as a better source for fermentation process because of covalent binding, efficient circulation of air and proficient infiltration by mycelia of fungal species. It is also economically beneficial substrate used in fermentation industry (Khan & Mubeen, 2012).

In the production of bioenergy, usage of immobilized enzymes on different types of nanoparticles has rigorously improved the procedure's practical viability and experimental value. Immobilized enzymes are more beneficial than free cells because they are thermo-stable, reusable, easy to store and also giver larger surface area-volume ratio (Ansari & Husain, 2012; Misson *et al*., 2015). These magnetic nanoparticles have inherent biocompatibility and larger surface-volume ratio (Perez *et al*., 2002) and they are widely applied in the field of environmental science and biomedical industry including drug delivery, magnetic bio-separation, destruction of tumor cells and pollution remediation (Telling *et al*., 2009; Wang & Irudayaraj, 2010).

Silica based nanoparticles functionalized with methyl-group exhibited best production of bioethanol (Kim *et al*., 2014). One of the drawbacks of nanoparticles usage is difficult to recover them for reuse. A complicated purification method and high performance centrifuge technique is required to recover nanoparticles from nanoparticles-culture broth mixture (Nemati *et al*., 2014). However, a cost effective and easy method is needed to recover nanoparticles economically. Magnetic nanoparticles provide the solution of easy recovery or nanoparticles

(Khaligh & Shirini, 2013). Iron nanoparticles have been studied in many fields and are most studied nano material. They find their applications in the field of cell biology, mining, environmental remediation, diagnostic and analytical chemistry (Ngomsik *et al*., 2009).

For efficient production of ethanol and rapid fermentation the yeast strain used should possess the qualitative and quantitative strength in ethanol production with significant growth rate in high ethanol concentration (Bai *et al*., 2008). There are several microorganisms that have capability to ferment sugars to obtain bioethanol but *Z. Mobilis* is considered as best known bacteria while *S. cerevisiae* is best known yeast in production of ethanol (Talebnia *et al*., 2010). *Saccharomyces cerevisiae* has capability of utilizing monosaccharide as well as polysaccharides making itan efficient microorganism to be used in variety of substrate (Badotti *et al*., 2008). The aim of this study was to enhance production of ethanol using immobilized yeast cells (*S. cerevisiae*) in Ca alginate and Ca alginate magnetic Nanoparticles (Ca-MNP). Different concentration of alkaline pre-treatment were applied on wheat straw to enhance the enzymatic saccharification. The reusability of magnetic nanoparticles was also studied.

Materials and Methods

The chemicals carried out for research work were of analytical grade and applied directly without purifying them further. Many of the chemicals used were obtained from Merck Germany/Sigma Aldrich. Some of chemicals were taken from Pakistan Institute of Industrial and Scientific Research of Department Food and Biotechnology Research Centre, Lahore? for microbial studies and preparation of culture media.

Substrate: Wheat straw as Lignocellulosic biomass was utilized and purchased from local markets of Pakistan. It was washed to remove irrelevant material and dried at 70^0C, transferred to plastic bags. Hammer beater was used to convert wheat straw into powder form of size 2mm. This powdered substrate was stored in polythene bag for LAB scale testing.

Pre-treatment: 100g sample of wheat straw was added in 1000ml conical flask and then soaked with different concentrations of NaOH including concentrations of 1.5%, 2.5%, 3.5%, 4.5%. In 1.5% of NaOH means 15g in 1000ml of water at room temperature for 1 hour. This substrate was autoclaved for 90 minutes at 121ºC. After that, substrate was washed under tap water 7-8 times by using muslin cloth. Then pH was neutralized by washing with distilled water (2-3times). Then sample was kept for overnight at 60 to 80ºC. The substrate was grind to fine powder by using hammer beater mill. Afterwards, the powdered substrate was stored in polythene bags (Lynd *et al*., 2002).

Enzymatic hydrolysis: Commercial cellulase enzyme was used for enzymatic hydrolysis to degrade residual substance collected after treatments. 2000ml conical flask was used and 1000ml acetate buffer with pH 5 was added. Additionally, 40g of pre-treated wheat straw was added along with 50g of commercial cellulase enzyme 6000u/g. Then, it was placed on shaker for 7 days at 30ºC with in speed of 150rpm for saccharification. Sugar release was calculated on daily basis by samples drowning out on every day.

Microorganism, media and inoculum preparation: *S cerevisiae*SS-4 yeast strain, used for fermentation process, was obtained from FBRC, PCSIR. The *S. cerevisiae*SS-4 was maintained in YPG agar medium (pH 4.5), containing yeast extract (3.0 g/L), peptone (5.0 g/L), glucose (10.0 g/L), agar (20.0 g/L).

Immobilization

Immobilization of yeasts in Ca alginate: 1g of calcium alginate was dissolved in 90.0ml distilled water. The yeast cell mass was obtained from 100 ml of culture broth. The cell-alginate solutions were completely mixed and added through a sterile needle to a stirred solution of 0.1 M $CaCl_2 2H_2O$ using a magnetic stirrer (Kostov *et al*., 2010). The beads were formed, stored at room temperature for 1.5 hr. and washed with sterile distilled water. The beads average size was 1.0 - 1.5 mm.

Ca alginate magnetic nanoparticles beads: Calcium alginate solutions and yeast cell slurry was mixed with 3% (w/v) dried iron nanoparticles, final calcium alginate concentration and final magnetic nanoparticles content was 5-20% (w/v). This mixture was stirred gently and chilled, adding drop wise into 2% (w/v) $CaCl_2.H_2O$ and kept for 2 hours for stabilization. Magnet was used to separate iron nanoparticles and distilled water was used along buffer and stored for two months in saline solution at 4°C to check the fermentation efficiency of immobilized cells after storage. After every 5 days, they were used for continuous process for 60 hours after that it was washed and stored again (Ivanova *et al*., 2011).

Fermentation medium: The hydrolyzate was supplemented with nutrients 1% yeast extract, 2% peptone, 5% glucose and sterilized at 10 psi for 20 min. Stirred tank fermentor (EYELA MBF 250, Japan) of 2.0 Litre with 75% working volume was used for fermentation. The ratio of fermentation medium and inoculums was set as 8:1 (1 ml inoculums contained 0.1 g immobilised yeast). For all the runs impeller speed was adjusted at 150rpm. The pH of sample was adjusted 6.5 and fermentation was carried away at 30ºC. After extraction, Whatman filter paper was used to remove ethanol from residue. Further distillation was done utilising rotary evaporator. Afterwards, sample was heated to get bioethanol at 80^oC.

Analytical methods

Sugar estimation: Total reducing sugar estimation was done by using dinitrosalisyclic acid method of Miller (1959) and Ingle (Ingle *et al*., 2017). Transmittance% was measured by using UV-Visible spectroscopy (Shimadzu UV-2800, Japan). Total sugar was estimated by following the method of Periyasamy *et al*., 2009.

Determination of cellulose, lignin and saccharification: Cellulose content in treated and untreated samples was calculated by using procedure described by Gopal & Ranjhan (1980). The lignin content was estimated by the procedure described by Milagres (1994). Saccharification (%) after enzymatic hydrolysis was calculated as described by Uma *et al.*, (2010).

$$\text{Saccharification (\%)} = \frac{\text{Reducing sugar formed x 0.9}}{\text{Cellulose content in pretreated biomass}} \text{ x } 100$$

Moisture content/ weight loss and ash Content: Moisture content was determined by oven dry method. The sample of known weight was placed in oven for 1 hour at 105ºC. Afterwards, the sample was weighed again. (Irfan *et al*., 2011). The 2g of sample was heated at for 4 hours at 550^0C in the furnace to determine ash content and desiccated to measure the weight of ash after cooling (Jittabuta, 2015). The Ash Content percentage was determined using following formula:

$$AC(\%) = \frac{B}{A} \times 100$$

where: B = weight of ash (g); A = Weight of sample before putting in furnace (g); AC = Ash content (%)

Ethanol estimation: The ethanol content was estimated by using the dichromate-sulphuric acid method of Semichon & Flanzy (1929) and fermentation efficiency (%) was determined as (Sharma *et al*., 2007):

$$FE(\%) = \frac{AEC}{TEC} \times 100 \quad (1)$$

$$TEC = TFS \times 0.51 \quad (2)$$

where: FE = Fermentation efficiency; AEC = Actual ethanol content; TEC = Theoretical ethanol content; TFS = Total fermentable sugars

Results and Discussion

Effect of pre-treatment: Pre-treatment of any lignocellulosic biomass is crucial before enzymatic saccharification (Saha, 2003). The method of pre-treatment had a pronounced effect on the yield of reducing sugars in the case of wheat straw. In this study 1.38 mg/mL reducing sugar was achieved at 2.5 % NaOH (Fig. 1), while with the increase of alkaline concentration reducing sugar concentration was decreased. While 66.23% cellulose and 46.52% weight loss was achieved at 1.5 and 2.5% NaOH respectively (Fig. 2). Maximum delignification (58%) was observed with 2.5% (v/v) NaOH. With increase in concentration of NaOH decline in cellulose content and increased weight loss was observed. Our results are in consistence with findings of Irfan (Irfan *et al*., 2011). Crystalline index decreases due to alkaline pre-treatment and specific area of biomass conversely increases. As a result, lignin undergoes unfolding and structure of lignin is changed (Zhang & Lynd, 2004; Zhang & Shen, 2006; Xu *et al*., 2010). Cellulose structure get changed with its morphology and crystalline structure also altered by alkaline reagents. This results in crystal saponification together with hydrolysis of polysaccharides chain (Cheng *et al*., 2010; Ibrahim *et al*., 2011; Sills & Gossett, 2011). Resultantly, the availability of monosaccharide increases and biomass density is lowered as hydrolysis proceeds (Shi *et al*., 2018; Hassan *et al*., 2018). Results of Asghar *et al*., (2015) revealed that 2.5% NaOH concentration is very important in achieving a maximum cellulose, delignification and hemicellulose content of 83%, of 81% 10.5% respectively. (Table 1) is showing proximate analysis of wheat straw in controlled and pre-treated sample with 2.5%NaOH.

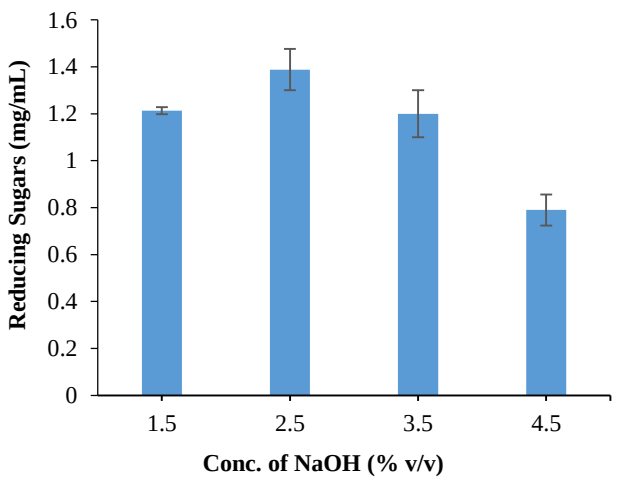

Fig. 1. Effect of different conc. of NaOH on reducing sugar (mg/mL) of wheat straw.

Table 1. Proximate analysis of wheat straw.

Wheat straw content	Control	Pretreated with 2.5%NaOH
Cellulose	40% ± 0.32	63% ± 0.52
lignin	19% ± 0.25	3% ± 0.921
Moisture	10% ± 0.094	7.40% ± 0.085
Ash	7.40% ± 0.34	2.80% ± 0.57

± Indicates Standard Deviation

Enzymatic saccharification: The alkali pre-treatment can result in a sharp increase in saccharification, with manifold yields (Kassim & El-Shahed, 1986). In this study, pre-treated wheat straws were used for 36.31% saccharification by utilizing commercial enzyme (Fig. 3). According to findings of Irfan *et al*., (2011) commercial cellulase enzyme was used in pre-treated wheat straw and sugarcane bagasse in saccharification of yield 33.6% and 63.3% respectively. But low level of saccharification was observed in indigenously produced cellulase enzyme which is up to 6-14% (Irfan *et al*., 2011). In present work amount of reducing sugar released was 0.37 mg/mL in five hours of hydrolysis and it remain almoststable in last two hours of observations while in pre-treated wheat straw 143.34 mg/mL of reducing sugar was found after seven h of hydrolysis (Fig. 4). Same result was observed with the increase of days (Table 2). Pre-treatment affectively increased the yield of hydrolysis process from 90% to 20% (Hamelinck, 2005).

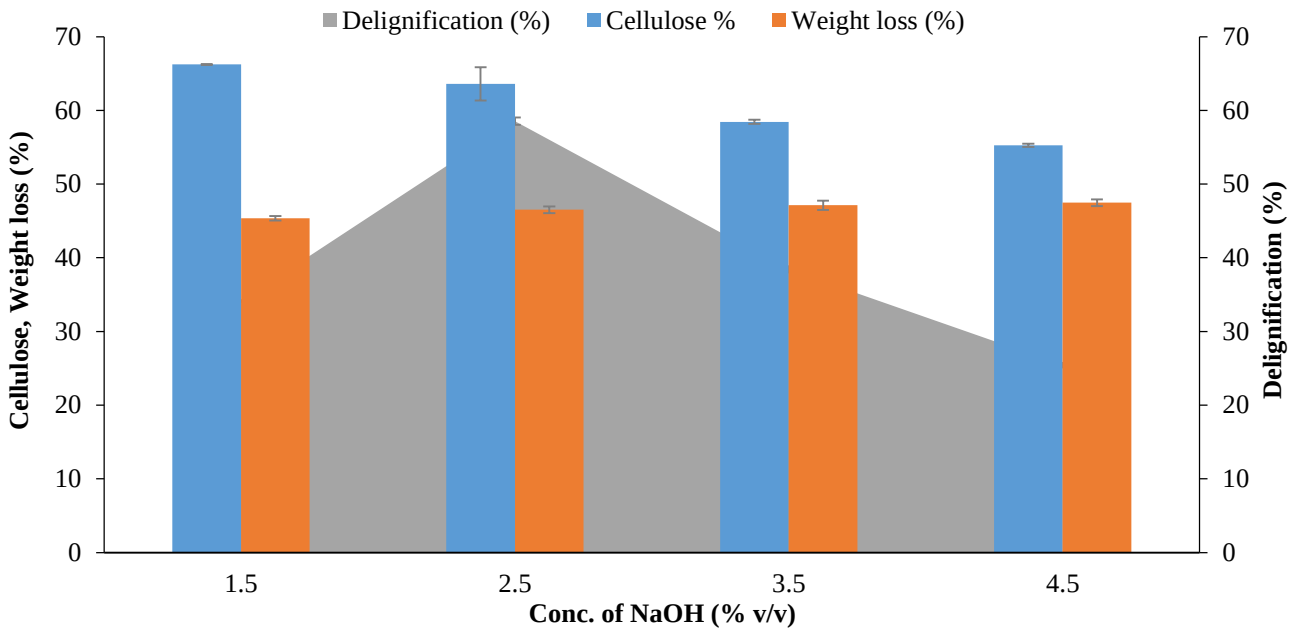

Fig. 2. Effect of different Conc. of NaOH on cellulose, weight loss and delignification of wheat straw.

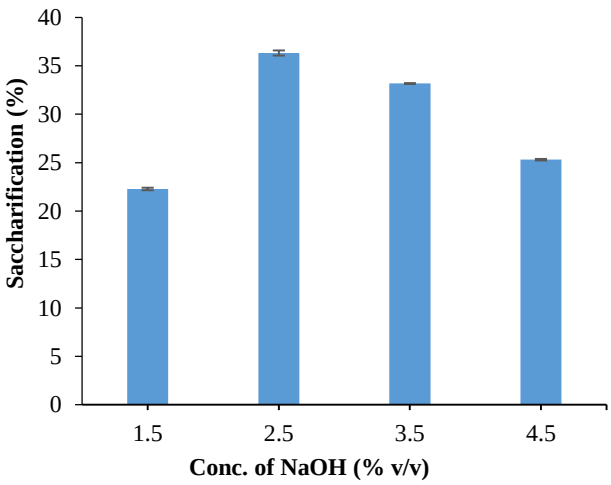

Fig. 3. Effect of different conc. of NaOH on saccharification of wheat straw.

Table 2. Saccharification of Pre-treated wheat straw using commercial enzyme.

Days	Reducing sugar mg/ml
1	220.45 ± 0.050
2	260.12 ± 0.051
3	310.30 ± 0.072
4	347.46 ± 0.399
5	400.27 ± 0.065
6	421.46 ±0.055
7	485.12 ± 0.072

± Indicates Standard Deviation

Ethanol production using *S. cerevisiae* SS-4 cells immobilized in Ca alginate and nanoparticles: Immobilized technique has been proved as an effective source of enhancing ethanol yield, since it is efficient to reduce cost and time for ethanol production and maximize rate of production (Durham, 1994). Ethanol yield was compared by using *S. cerevisiae* SS-4 cells immobilized in Ca alginate, *S. cerevisiae* SS-4 cells immobilized in Ca-MNP with *S. cerevisiae* SS-4 free cells (Fig. 5). Ethanol production rate was higher in immobilized cells as compared to free cells. Jianliang concluded that immobilized yeast cells produce more ethanol rather than free cells (2.24 times more ethanol) (Jianliang *et al*., 2007). Growth of yeast cells in gel is higher as compared to growth of free yeast cellsand ethanol production capacity of immobilized cells is also higher (Wada *et al*., 1980). Similarly, Galazzo & Bailey (1990) examined that yeast cells grown in alginate matrix produced high yield of ethanol. This yield was 50% greater than ethanol produced in free suspended cells.

Literature studies also revealed immobilization of microorganism in nanoparticles to produce bioethanol. Kim *et al*., in 2014 investigated that $CoFe_2O_4$ @ SiO_2-CH_3 nanoparticles enhance the productivity of biomass, ethanol and acetic acid by 213.5%, 213.5%, and 59.6 % respectively. It was observed that ethanol production from *S. cerevisiae* SS-4 cells immobilized in Ca alginate was 45.66 g/L, while ethanol production*in S. cerevisiae* SS-4 cells immobilized in Ca-MNP was higher i.e., 49.71g/L (Fig. 5). It might be due to utilizing alginate based carriers for example restriction in mass transfer, gel degradation and less physical strength. There are reports that yeast cells growth in immobilization is lower because entrapment in Ba-alginate gel cause lower oxygen diffusion (Dias *et al*., 2001; Bangrak *et al*., 2011). Our result statements were in accordance with other research workers that reported iron nanoparticles in yeast cells act as cofactor for many enzymes. Iron is a limiting factor and its availability is very important for growth of yeast cells. It is involved in metabolic processes and synthesis of chytochrome. Ethanol production by using *S.cerevisiae* is very effective technology in term of higher production (Najafpour *et al*., 2004).

Reuse of immobilized cells: Nanoparticles were recovered and used again in the process of fermentation to confirm its reusability. Ivanova *et al*., (2011) reported yeast cells immobilized in saline solution at 40ºC are stable for more than one month. Cells visibility and stability was retained by Ca alginate and Ca-MNP synthesized with fixed yeast cells and maximum formation of ethanol in first cycle was produce. The results show the ethanol production of *S. cerevisiae* SS-4cells immobilized in Ca alginate was 44.8g/L in first cycle and 31.01g/L in fifth cycle. Same trend was observed by reuse of *S. cerevisiae* SS-4cells immobilized in Ca-MNP (Fig. 6). It showed that productivity of immobilized cells in all cycles was profitable, same results were obtained by others. According to the study by Ivanova *et al*., (2011), bioreactor utilizing immobilized *S. cerevisiae* cells carried out the fermentation process for above 42 days without significant loss in ethanol production. In the study conducted by Zhao and Xia, immobilized yeast produced ethanol with concentration of 30.1g/l in 5 batch fermentation (Zhao & Xia, 2010). According to findings of Galazzo & Bailey (1990) yeast cells could be used eight times and immobilization barrier provide protection from contamination.

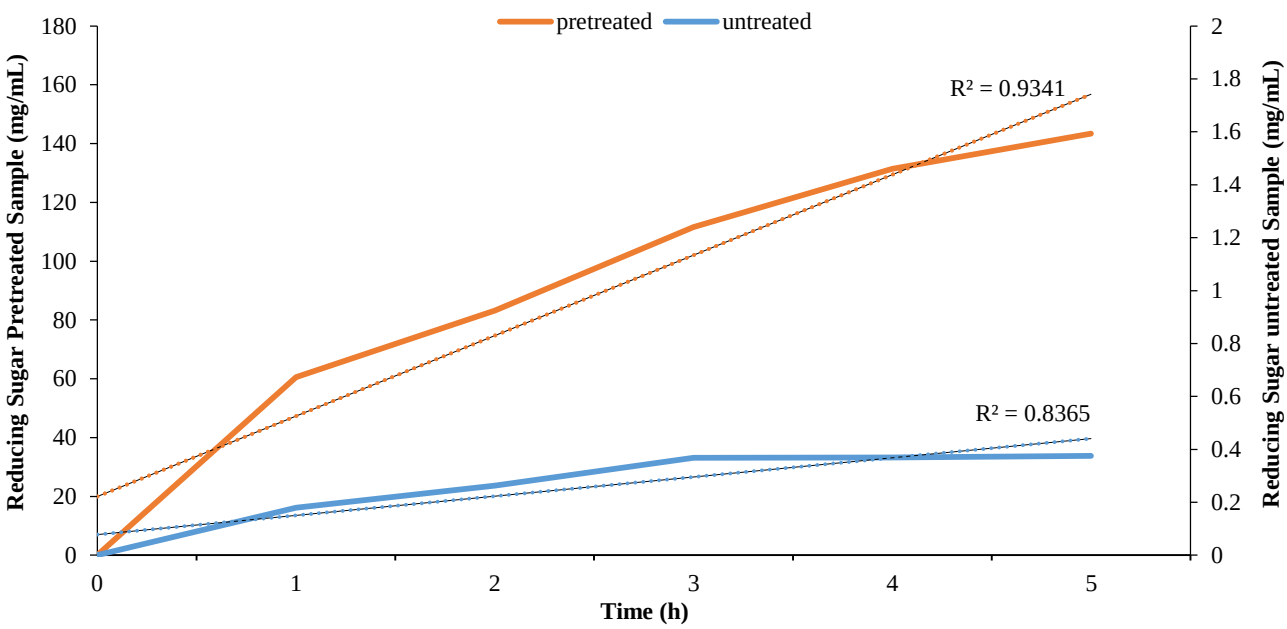

Fig. 4. Sugar released during enzymatic hydrolysis.

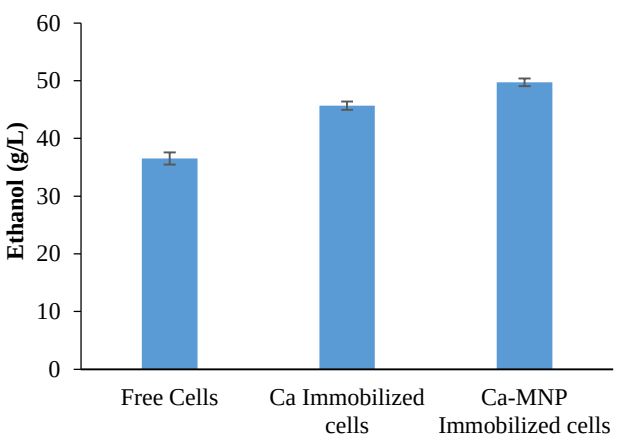

Fig. 5. Comparison between immobilized cells and free cells on ethanol production using pre-treated and hydrolysed wheat straw.

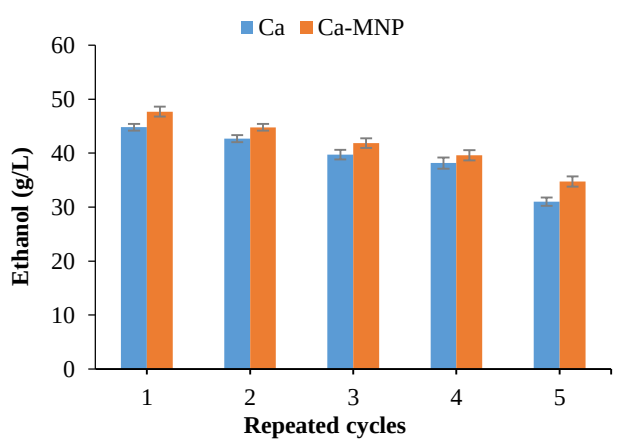

Fig. 6. Effect of reuse of immobilized cells on ethanol production using pre-treated and hydrolysed wheat straw.

Conclusion

Today world is facing three critical problems high fuel prices, climatic changes and air pollution; to overcome these problems bioethanol by using lignocellulosic materials is best choice. Pre-treatment of any lignocellulosic biomass is crucial before enzymatic saccharification. Immobilized cells are more efficient to reduce cost and time for ethanol production and maximize rate of production. *S. cerevisiae* SS-4 cells immobilized in magnetic nanoparticles (MNP) showed more efficiency in producing ethanol in less time than the yeast cells immobilized on Na-alginate. Batch fermentation in repeated cycles, immobilized yeast cells showed high productivity making it profitable process additionally reducing the time of inoculums preparations by enhancing ethanol production.

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