EVALUATION OF CARROT GRASS (PARTHENIUM SP.) FOR ETHANOL PRODUCTION

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EVALUATION OF CARROT GRASS(*PARTHENIUM SP.*) FOR ETHANOL PRODUCTION

By

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MY WIFE
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1. INTRODUCTION

The world’s economy is mostly dependent on various fossil sources such as coal, oil, natural gas, etc. for energy. These are being used for the production of fuel, electricity and other goods (Uihlein and Schbek, 2009). Due to the ever increasing human population and industrial development, the global energy demand has increased tremendously. India has 0.5% of the oil and gas resources of the world and 16 % of the world’s population, thus require huge amounts of oil imports to meet the domestic demand (Sukumaran and Pandey, 2009). More than 70 % of the needs of the country are met from imports and the demand for gasoline has been growing at an average rate of approximately 7% per annum during last decade (MPNG, 2009). In the near future the import of crude oil will be affected due to limited non renewable reserves, in such a scenario, renewable biomass might serve as an alternative resource for biofuel production.

The Government of India launched National Biofuel Mission in 2003, mainly with a perspective of exploring the potential of biofuel as a cleaner source of energy and to partially offset the growing burden of crude oil import bills. The National Biofuel Policy (2009) released by Ministry of New and Renewable Energy (MoNRE) envisages a target of complete blending of 5% ethanol in the coming future which will require 0.64 billion liters of ethanol. Further it will be gradually raised to 10% by 2016-17 and to 20% after 2017. Most of the major biofuel producing countries have exploited the staple food crops for the production of bioenergy and it is estimated that, about 60% of ethanol produced, is generated from cereals and sugarcane, both of which are important food crops. The diversion of food crops for energy production was one of the major factors for drastic rise in food prices in international market (von Braun, 2008) which urged to the need to shift to greener non-food energy sources.

Lignocellulosic biomass is among the major renewable resources which may be exploited for second generation biofuel, without competing with food crops (Sukumaran et al., 2010). It has been estimated that 442 billion litres of bioethanol per year could be produced from lignocellulosic biomass across the world, about 16 times higher than the actual world bioethanol production. Also, the production of bioethanol could be an effective way for utilization of agricultural wastes and reduction of environmental pollution (Kim and Dale, 2004). Rice straw, wheat straw,
corn straw, and sugarcane bagasse are the major agricultural residues available for biofuel production. However, all these lignocellulosic biomass have other competitive uses as feed or fuel. Therefore, it is important to find alternative renewable lignocellulosic biomass which can be utilized as the potential feedstock like weeds for bio-fuel generation.

Weedy lignocellulosic biomass may be explored for production of second generation biofuel due to their growth sustainability and year round availability. *Eichhornia crassipes, Lantana camara, Prosopis juliflora, Saccharum spontaneum, Crofton and Chromolaena odorata* etc. are some of the most common weedy feedstocks explored for their efficiency to produce bioethanol (Chandel et al., 2011; Huber and Dale, 2009). *Lantana camara* and *Parthenium hysteroporous* are two important weed species available throughout the India. The approximate biomass produced by *L. camara* over a year ranges from 15 to 17 tonnes/ha (Bhatt et al., 1994) and it contains 61% holocellulose and being explored for bio-ethanol production by Kuhad et al. (2010).

*Parthenium* sp. (commonly known as “congress/carrot grass”) is an aggressive noxious perennial herbaceous weed and a prolific seed producer. *Parthenium* weed was first noticed in India in Maharashtra which has spread throughout the subcontinent nearly over 35 million hectares of land leading to 40% loss in terms of economic yields (Sushilkumar, 2009; Swati et al., 2013). It has been reported that population density of *Parthenium* sp. ranges from 4-168/m² and fresh weight from 1-40 tonnes/ha (Saravanane et al. 2012). Therefore, if *Parthenium* can be used as a feedstock for biofuel production, two advantages can be envisaged viz. effective management of unwanted and harmful weed biomass and production of ethanol.

The conversion process of lignocellulosic biomass into fermentable sugar is still technically problematic as the digestibility of cellulose is masked by recalcitrant lignin. Pretreatment is an essential step which breaks down the lignin structure and disrupts the crystalline structure of cellulose and thus increase accessibility for hydrolytic enzymes (Moiser et al., 2005). Since different lignocellulosic biomass have different physico-chemical characteristics, it is important to adopt a suitable pretreatment technology based on the properties of each raw material (Galbe and Zacchi, 2007). Among various pretreatment technologies, alkali treatment which increases the cellulose digestibility and effectively solubilises the lignin with minor
solubilisation of cellulose and hemicelluloses is most widely used (Kumar et al., 2009a). Acid treatment solubilises the hemicellulosic fraction of the biomass and increase the cellulose accessibility to enzymes (Alvira et al., 2010). Biological treatment with white rot fungi which preferentially degrade lignin (Saritha et al., 2012; Tiwari et al., 2013; Rana et al., 2013) is also being reported as suitable options for biofuel production. The pretreated biomass may further be saccharified in to soluble sugar by using commercial cellulase enzyme preparation.

Glucose and xylose are the two major sugars in the lignocellulosic hydrolysates (Chandrakant and Bisaria, 1998) and there is no single organism that can efficiently ferment both these sugars to ethanol (Fu and Peiris, 2008). For the overall utilization of the sugar in saccharified material, a co-culture system in which potential glucose-fermenting yeast is cultivated with a xylose-fermenting yeast is useful (Taniguchi et al., 1997). Saccharomyces cerevisiae, the widely studied yeast for hexose fermentation, is unable to utilize pentose sugars, while among the pentose fermenting yeasts Pichia stipitis, Candida shehatae and Pachysolen tannophilus have proved to be highly efficient (Abbi et al., 1996). Simultaneous conversion of glucose and xylose to ethanol using co-culture of hexose and pentose fermenting yeasts results in high ethanol yields from lignocellulose as almost all the sugars are converted to ethanol (Yadav et al., 2011). Therefore, a highly effective pretreatment method coupled with co-fermentation of hexoses and pentoses with efficient yeast strains can be a promising option for achieving higher ethanol yields.

Keeping the above concerns in consideration, the present research work has been proposed with the following objectives:

- Bioprospecting of microorganisms for delignification and saccharification of lignocellulosic biomass
- To develop a biological delignification process for carrot grass biomass
- To standardize an enzymatic method for conversion of delignified carrot grass biomass to fermentable sugars
- To develop a fermentation process for bio-ethanol from sugar hydrolysate of carrot grass biomass.
2. BACKGROUND

The search for alternative sources i.e. biomass resources assume importance due to the soaring crude oil price and depletion of fossil fuel reserves coupled with the rising environmental concerns. This has created interest in the production and use of fuels from plants or organic waste which ultimately reduce both the dependence on oil and air pollution. Biofuel is very important for rural and urban areas in terms of energy security, environmental concern, agricultural development, employment opportunities, socio economic issues etc. The first generation biofuel is produced mainly from sugarcane molasses and starchy biomass like corn, tubers and grains. Annual commercial production of ethanol from these sources accounts almost 50 billion litres (Naik et al., 2010). But there are concerns about its impact on biodiversity, land use, competition with food crops and sustainability. The cumulative impacts of these concerns have increased the interest in developing second generation biofuels from non-food biomass i.e. lignocellulosic biomass.

Lignocellulosic biomass is considered as the only foreseeable, feasible and sustainable resource for renewable fuel as these raw materials do not compete with food crops and are less expensive than conventional agricultural feedstock. It includes cereal straw, bagasse, forest residues and purpose-grown energy crops such as vegetative grasses. Ethanol from renewable lignocellulosic biomass of non-edible crops has been identified globally as the future solution to meet the future energy demand. These second generation biofuels could avoid many first generation problems and potentially offer greater cost reduction in the long term. Annual production of lignocellulosic biomass is estimated to be $1 \times 10^{10}$ MT worldwide (Sánchez and Cardona, 2008).

2.1 Lignocellulosic biomass

Biomass is produced by green plants through conversion of sunlight into plant material and includes all land and water based vegetation, as well as all organic wastes. Lignocellulosic biomass is a renewable organic material and is the major structural component of plants (Fig.1). It consists mainly of cellulose (35-55%), hemicelluloses (20-40%) and lignin (10-25%) (Ghosh and Ghose, 2003).
2.1.1 Cellulose

Cellulose is the major constituent of all plant material and the most abundant organic molecule on earth. Cellulose is a linear polysaccharide, made by the polymer of glucose units which are linked by β-1, 4 glycosidic bonds. The cellulose chains are interlinked by hydrogen bonds in so-called ‘elementary microfibrils’ and these fibrils are attached to each other by hemicelluloses, amorphous polymers of various sugars and also other polymers such as pectin, and covered by lignin. This complicated structure makes the cellulose resistant to various pretreatments (Ha et al., 1998).

2.1.2 Hemicellulose

Hemicellulose, the second most abundant component of lignocellulosic biomass, is a heterogeneous polymer of hexoses (mainly mannose, less glucose and galactose), pentoses (including xylose and arabinose) and acids of sugars. Hemicellulose concentration in lignocellulosic biomass is 25 to 35% and it is easily hydrolysable to fermentable sugars due to its less complexity (Saha et al., 2007). Mannose and xylose are the dominant sugars of hemicellulose in soft woods and hard woods, and agricultural residues, respectively (Persson et al., 2006; Balan et al., 2009).

2.1.3 Lignin

Lignin is ranked as the third main heterogeneous polymer in lignocellulosic biomass and generally contains three aromatic compounds: coniferyl alcohol, sinapyl alcohol and p-coumaryl alcohol. Lignin serves as a back bone to the biomass fibres for its structural strength and also it acts as a barrier to any enzymes or solutions due to its linkage with both cellulose and hemicelluloses and prevents penetration of lignocellulolytic enzymes to the interior of biomass structure. The most important characteristic of lignin is that it is the most recalcitrant component of lignocellulosic biomass (Zaldivar et al., 2001; Hamelinck et al., 2005).

2.2 Lignocellulosic substrates used for ethanol production

The polymeric material present in the lignocellulosic biomass is broken down into component sugars which are then fermented to ethanol. The process of hydrolysis of biomass to generate sugars is called saccharification. It can be performed either by chemical or biological means. Enzymes capable of breaking the complex biomass into simple units are employed. Both cellulose and hemicellulose
can be hydrolyzed to yield fermentable sugars which may be then fermented to ethanol by using appropriate microorganisms (Hahn-Hägerdal et al., 2006). Lignin cannot be used for the production of ethanol but can be used for other applications (Hu, 2002; Lora and Glasser, 2002).

Fig. 1 Molecular component of plant cell wall structure (Rubin, 2008)

The typical process for lignocellulosic ethanol production consists of biomass pretreatment and detoxification followed by hydrolysis, alcohol fermentation and product recovery (Margeot et al., 2009). The biomass is treated with either dilute acid or alkali at high temperature which helps in liberating the hemicellulose sugars or lignin, respectively, and makes the cellulose susceptible to enzymes in saccharification (Himmel et al., 2007; Kumar and Wyman, 2009a). Enzymatic saccharification of pretreated biomass is performed with a cocktail of enzymes having the cellulases as a major component. The sugar rich hydrolysate is then fermented to produce alcohol by using yeasts. The hemicellulosic fraction separated in acid pretreatment can be fermented by pentose fermenting microbes for production of alcohol (Mamman et al., 2008). Various bottlenecks in bioethanol production include, pretreatment of biomass, enzymatic saccharification of the pretreated biomass and fermentation of hexose and pentose sugars released during hydrolysis and saccharification. Each of these problems urges substantial R&D
efforts for improved efficiency and economics of the process (Sukumaran and Pandey, 2009a).

2.2.1 Weedy biomass for bioethanol production

The major types of biomass used for ethanol production are monoculture crops grown on fertile soils such as sugarcane, corn, soy beans, oilseed rape, switch grass and hybrid poplar (Farrell et al., 2006) and agricultural wastes such as rice and wheat straw, corn stover and waste wood (Kim and Dale, 2004). Another type of biomass is weedy cellulosics viz. *Eichhorniacrassipes*, *Lantana camara*, *Saccharumspontaneum*, *Typhalatifolia*, *Prosopisjuliflora*, *Chromolaenaodorata* etc. which are promising and cheaper feed stocks for production of fuel ethanol in future. These weedy substrates do not require additional economic input as they normally grow on agriculturally degraded land and water bodies (Huber and Dale, 2009).

*E. crassipes* (water hyacinth) is a free-floating perennial aquatic weed with long, spongy, and bulbous stalks. It is a vigorous grower that can double its population in two weeks and can serve as potential biomass for ethanol (Kumar et al., 2009a) and cellulase production (Sukumaran et al., 2009). *L. camara* is a noxious weed that can threaten land productivity, livestock and biodiversity. However, its luxuriant growth and vigorous survival makes it a potential source for utilization in value-added products such as ethanol (Pasha et al., 2007; Kuhad et al., 2010). *S. spontaneum* (wild sugarcane) is a perennial weedy grass, spread across millions of acres in India, often leading to abandonment of fields. It can be an excellent source for ethanol and cellulase production (Chandel et al., 2009b).

*P. juliflora* is a tree which grows up to 12 m, with a trunk of diameter up to 1.2 m and provides enough biomass for ethanol production (Gupta et al., 2009). Switch grass (*Panicumvirgatum*) is a native, tall (2-6 ft) prairie grass well known for its rapid growth during warm months in most parts of the United States. Its ability to produce high yields with low applications of fertilizer and other chemicals, and tolerance to poor soils, flooding and drought make it a suitable biomass for bioethanol production. *Miscanthusgiganteus* is another viable feedstock for cellulosic ethanol production, which is similar to switch grass in cold and drought tolerance and water use efficiency (Ng et al., 2010). It is commercially grown as a source for combustible energy in European Union.
One of the most common noxious weeds, *Parthenium* sp. invades all disturbed land, including farms, pastures and roadsides. The species *Parthenium hysterophorus*, also known as congress grass or gazar ghas, has become a common problem in India. Despite its abundant nature in the subcontinent, there has been no report yet on ethanol production from this weed (Chandel et al., 2011).

**2.2.2 Parthenium sp.**

*Parthenium hysterophorus* is an annual herbaceous plant with deep tap root and an erect stem that gradually becomes semi-woody with age. It can grow and reproduce itself any time of the year. Low temperature (<10°C) considerably reduces plant growth, mainly flowering and seed production. *Parthenium* is a prolific seed producer; a single plant can produce 2,00,000 seeds/m² in a highly infested field. It is able to grow in areas with summer rainfall greater than 500 mm per annum and germination is favoured at temperature between 10°C and 25°C. Many aspects of the ecology of *Parthenium* weed contribute to its aggressiveness which includes size, persistence and longevity of seed, fast germination rate and innate dormancy which makes it well adapted to semi-arid conditions (Swati et al., 2013).

*P. hysterophorus* is believed to have been introduced into India along with PL 480 wheat (Public Law 480) as contaminants when imported from USA in the 1950s. Presently, this invasive weed has infested about 35 million hectares of land in India since its first notice in 1955 (Sushilkumar, 2009).

**2.2.3 Harmful aspects of Parthenium**

The adverse effects of *Parthenium* sp. may be summarised under the following two aspects.

**2.2.3.1 Health hazards to humans and livestock**

*Parthenium* is known to cause many health hazards which have reached up to the epidemic level. Agriculturists are more concerned about *P. hysterophorus* affecting food and fodder crops, since the pollen and dust of this weed elicit allergic contact dermatitis in humans (Gunaseelan, 1987). Persons exposed to this weed for prolonged period develop the symptoms of skin inflammation, asthma, allergic rhinitis, hay fever, black spots, burning and blisters around eyes, and exposure to pollens causes allergic bronchitis (Towers and Subba Rao, 1992). In livestock, it causes systemic toxicity when exposed and the milk and meat quality of cattle,
buffalo and sheep deteriorate on consumption of this weed (Lakshmi and Srinivas, 2007).

2.2.3.2 Reduction in agricultural productivity and biodiversity

*P. hysterophorus* contains a bitter glycoside, parthenin, a major sesquiterpene lactone (SQL) which gets leached as root exudates and play a pivotal role in allelopathic interference with surrounding plants (Belz et al., 2007). Burning of this weed in fields result in reduced germination, biomass growth, chlorophyll content and poor fruiting (Lakshmi and Srinivas, 2007) and also play a role as alternate host for crop pests.

In *P. hysterophorus* dominated areas, very sparse and sometimes no other vegetation can be seen because of its rapid invasion and replacement of the indigenous species in the surroundings and hence, pose a serious threat to biodiversity in India. The more vigorous mode of reproduction and the possession of an array of secondary metabolites give the weed the status of invasive alien species (IAS) (Patel, 2011).

Introduction of competitive plants such as *Cassia sericea* and *C. tora* were recommended to manage *Parthenium* in wasteland, road sides and community land. Despite many reports of pathogens causing disease on *Parthenium*, none of them has qualified as a successful bioagent. Also not even a single indigenous insect species has proved successful in spite of their occurrence and infestation on *Parthenium*. Only a host specific leaf feeding beetle, *Zygogrammabicolorata*, is proved to be a successful biocontrol agent in reducing the *Parthenium* densities in different parts of India. *Parthenium* management through this bioagent cannot be done completely due to high regeneration capacity, germination ability, seed production ability and extreme adaptability of *Parthenium* in wide range of ecosystem. Also *Parthenium* is able to germinate and grow throughout the year (Sushilkumar, 2009). Therefore, *Parthenium* biomass seems to be a viable option for biofuel production in India.

2.3 Pretreatment methods for production of lignocellulosic ethanol

The most important processing challenge in biofuel production is pretreatment of the biomass. The basic objective of pretreatment is to make the lignocellulosic complex accessible to cellulase enzyme through solubilisation or removal of either hemicelluloses or lignin from the complex so that cellulose may be accessible for saccharification. The shielding of cellulose by hemicelluloses and
lignin in plant material hinders the process of hydrolysis (Kumar et al., 2009a). Goals of an effective pretreatment process are (a) to form sugars directly or subsequently by hydrolysis (b) to avoid loss and/or degradation of sugars formed (c) to limit the generation of inhibitory substances (d) to reduce energy demands and (e) to minimize costs. Physical, chemical, physicochemical and biological treatments are the four fundamental types of pretreatment techniques usually employed. Generally, a combination of these processes is used in pretreatment of biomass, since any single method is not effective for commercial scale up.

2.3.1 Physical pretreatments

Mechanical comminution and extrusion are the methods employed for physical pretreatment of lignocellulosic substrates.

2.3.1.1 Mechanical comminution

The main objective of this pretreatment is to reduce the particle size and lignocellulosic crystallinity in order to increase the specific surface area and to reduce the degree of polymerization. This can be achieved by grinding or milling to a final particle size of 10-30 mm to improve the enzymatic hydrolysis of lignocellulosic materials (Sun and Cheng, 2002; Taherzadeh and Karimi, 2008). But the power requirement is relatively high and the continuous rise of energy prices makes this method economically unfit (Hendriks and Zeeman, 2009).

2.3.1.2 Extrusion

Extrusion process is a promising and novel method for conversion of biomass into ethanol. In this method, the materials are subjected to heating, mixing and shearing which result in physical and chemical modifications during the passage through the extruder. The barrel temperature and screw speed are believed to disrupt the biomass structure and increases the accessibility of cellulose for enzymes (Karunanithy et al., 2008).

2.3.2 Chemical pretreatments

Chemical pretreatment methods include the organosolv process, ozonolysis, acid pretreatment and acid pretreatment.

2.3.2.1 Organosolv pretreatment

Organosolvation is a promising pretreatment strategy in which numerous organic or aqueous solvent mixtures can be utilized including methanol, ethanol and
acetone in order to solubilise lignin and provide cellulose accessibility for enzymatic hydrolysis. The main advantage of this method is recovery of relatively pure lignin as a by-product (Zhao et al., 2009). The only limitation in this method is that solvents need to be separated as they may act as inhibitors during hydrolysis and fermentation (Sun and Cheng, 2002) and the cost of solvents is high if applied at commercial scale. These factors need to be considered while scaling up this pretreatment for industrial applications.

2.3.2.2 Ozonolysis

Ozone is a powerful oxidant which shows high delignification efficiency (Sun and Cheng, 2002) and removal of lignin ultimately increases the sugar in enzymatic hydrolysis. As this treatment is usually performed at room temperature and normal pressure, it does not produce any inhibitory compounds that can affect subsequent saccharification and fermentation. An important drawback is requirement of large amounts of ozone which make the process economically unviable (Garcia-Cubero et al., 2009; Sun and Cheng, 2002).

2.3.2.3 Acid treatment

Acid pretreatment involves the use of concentrated and diluted acids to break the rigid structure of the lignocellulosic biomass. The most commonly used acid is dilute sulphuric acid (H$_2$SO$_4$), which has been commercially used for the pretreatment of wide variety of biomass (Alvira et al., 2010). This chemical pretreatment involves the addition of dilute acids (0.2 – 2.5%) to the biomass, followed by constant mixing at temperatures between 130°C and 210°C for a few minutes to hours (Sun et al., 2005; Saha et al., 2005). This method is used most widely for pretreatment of substrates. Hsu et al. (2010) showed a maximum sugar yield of 83% by sulphuric acid (1%) pretreatment at 160-180°C for 1-5 min, followed by enzymatic hydrolysis.

Saha et al. (2005) pretreated wheat straw with dilute H$_2$SO$_4$ (0.75%) in an autoclave at 121°C for 1 h and saccharified enzymatically using cellulase, β-glucosidase, xylanase and esterase at 45°C for 72 h. The maximum yield of monomeric sugars was 565 ± 10 mggds$^{-1}$ and the yield of ethanol from the hydrolysate (78.3 g/L) was 19 ± 1 g/L with a yield of 0.24 g/g dry solid. Sindhu et al. (2011) studied the feasibility of using sugarcane tops as feedstock for bioethanol production. The substrate pretreated with 2% H$_2$SO$_4$, heated at 121°C for 60 min
produced 0.685 g of reducing sugar per gram of pretreated biomass under optimum conditions (11.25% solid loading, 50 FPU, 0.2% surfactant and 60 h incubation time). The fermentation of the hydrolysate using *Saccharomyces cerevisiae* produced 11.36 g/L of bioethanol with an efficiency of about 50%.

Kumar et al. (2009b) has investigated water hyacinth (*Eichhornia crassipes*) acid hydrolysate for ethanol production. The acid hydrolysate was produced by treating the substrate with 10 volumes of (2% v/v) sulphuric acid for 7 h at room temperature, stirred at 250 rpm and the hydrolysate was filtered to remove the solid residues. Fermentation of acid hydrolysate resulted in 72.83% conversion of xylose to ethanol with a yield of 0.425 g/g and productivity of 0.176 g/L/h. Hernandez et al. (2013) pretreated empty pods of *Moringa oleifera* fruits with dilute sulphuric acid (10% substrate loading) at 130-190°C for 10-30 min. Overall cellulose recovery was above 95% under 130 and 160°C but the highest sugar concentration (15.0 g/L) was obtained in the pretreatment performed at 160°C and 20 min.

### 2.3.2.4 Alkali treatment

Alkali pretreatment employs bases such as sodium, calcium and ammonium hydroxides which degrade ester and glycosidic side chains resulting in structural alteration of lignin, swelling and partial decrystallization of cellulose (Cheng et al., 2010). Sodium hydroxide has been extensively studied for many years and has shown disruption in lignin structure of biomass and increase in the accessibility of enzymes to cellulose and hemicellulose (Zhao et al., 2008a; Zhu et al., 2010). The alkali treatment involves soaking the biomass in alkaline solution and mixing it for certain period of time at specific temperature. Also neutralization of treated biomass is necessary to remove lignin and inhibitors before enzymatic hydrolysis. Another method is using lime as it has the advantage of low cost among the alkali treatments. It removes lignin and ultimately increases enzyme effectiveness by reducing nonproductive adsorption sites and increasing cellulose accessibility (Kim and Holtzapple, 2006).

Ko et al. (2009) pretreated rice straw with aqueous ammonia solution at moderate temperatures (50-70°C) for the production of maximum amount of fermentable sugars. Optimal conditions [69°C, 10 h and 21% (w/w) ammonia] resulted in 71.1% enzymatic digestibility of rice straw. Sorghum straw pretreated
with 2% NaOH at 121°C for 60 min resulted in 533 mg of glucose, 234 mg of xylose and 29 mg of arabinose per gram of washed solids and 135 mg/g of acid-insoluble lignin was recovered as precipitate (McIntosh and Vancov, 2010).

Pretreatment with calcium hydroxide (0.075g/ g dry biomass) increased the enzymatic hydrolysis of corn stover nine times compared to untreated, when loaded with 10 FPU/ g dry biomass and hydrolysed at 40°C. The enzymatic conversion of the corn stover was 60% cellulose and 47% xylan and 53% total available polysaccharide (Kaar and Holtzapple, 2000). Kim et al. (2003) pretreated corn stover with aqueous ammonia in a process termed ammonia recycled percolation (ARP). This pretreated substrates resulted in 70-85% reduction in lignin content and 40-60% hemicelluloses solubilisation, but left cellulose intact. The enzymatic digestibility was 99% with 60 FPU/g of glucan enzyme loading when treated for 90 min.

Li et al. (2012) treated corn stover with NaOH, NaOH + anthraquinone (AQ), NaOH + Na₂SO₃(alkaline), NaOH + Na₂SO₃(neutral), and NaOH + Na₂S. Among various treatments, alkaline sodium sulphite pretreatment removed 92% lignin at relatively low temperature of 140°C for 30 min with total sugar yield of 78.2% in enzymatic hydrolysis. This yield was 24% higher than that achieved with NaOH only under same conditions. Wang et al. (2012) studied that the pretreatment of conventional and genetically transformed switch grass (Panicumvirgatum L.) with 0.5%, 1%, 2% (w/v) NaOH at 121°C for 15, 30, and 60 min, respectively, followed by enzymatic hydrolysis with commercial cellulases and xylanases. At optimal conditions (1% NaOH, 30 min), the conversion efficiency of glucan and xylan from transgenic plants were 16% and 18% higher than the conventional plant, respectively.

Chaudhary et al. (2012) has optimized different alkaline pretreatment methods (NaOH, NaOH + 10% urea and aqueous ammonia) for maximum delignification of Saccharumspontaneum at 30°C followed by acid (H₂SO₄) hydrolysis (60% v/v) to solubilise cellulose and hemicellulose from solid residue. The slurry was diluted to a final concentration of 10% (v/v) and kept at 100°C for 1 h. It has been shown that the best result was 0.58 g (85%) reducing sugars/g of initial biomass after acid hydrolysis of aqueous ammonia pretreated biomass and ethanol yield of 0.35 g/g. Xu et al. (2010) explored switch grass to improve its enzymatic digestibility with lime loading of 0.1 g/g of raw biomass at 50°C for 24 h. The yield
of glucose and total reducing sugars reached 239.6 and 433.4 mg g\(^{-1}\) raw biomass, respectively, which were 3.15 and 3.61 times those of untreated biomass.

Gupta et al. (2011a) studied the effectiveness of alkali, acid and chlorite pretreatment by using the feedstocks such as corncob, *Prosopis juliflora* and *Lantana camara*. They were pretreated with various concentrations of sulphuric acid, sodium hydroxide and sodium chlorite at 121°C for 15-60 min. Among these treatments, chlorite pretreatment removed maximum lignin with approx. 90% (w/w) residual holocellulose which were enzymatically saccharified to yield 86.4% to 92.5% reducing sugars.

### 2.3.3 Physico-chemical pretreatments

Physico-chemical pretreatment of lignocellulosics may be accomplished by the following methods.

#### 2.3.3.1 Steam explosion

Steam explosion is the most widely employed physico-chemical pretreatment for lignocellulosic biomass. It combines mechanical forces and chemical effects due to the hydrolysis of acetyl groups present in hemicellulose, which takes place when high temperatures promote the formation of acetic acid from acetyl groups. The hemicellulose is partially solubilised and also the lignin is removed to some extent from the material (Pan et al., 2005). This ultimately exposes the surface of cellulose which increases enzyme accessibility to cellulose microfibrils. It has been successfully performed with agricultural residues and herbaceous biomass for ethanol production (Sun and Cheng, 2002). The main disadvantage of this pretreatment is partial degradation of hemicelluloses and generation of some toxic compounds that could affect hydrolysis and fermentation steps (Oliva et al., 2003).

#### 2.3.3.2 Liquid hot water

Liquid hot water is another hydrothermal treatment which does not require any catalyst or chemicals. Pressure is applied to maintain the water in liquid state at elevated temperatures (160-240°C) which provoke alterations in the structure of the lignocellulose. The main objective of this treatment is to solubilise mainly the hemicellulose and make the cellulose more accessible to enzymes and avoid the formation of inhibitors and it has been shown to remove hemicellulose up to 80% with enhanced enzymatic digestibility of pretreated material in herbaceous
feedstocks (Moiser et al., 2005a). The high water demand and energy requirement in this process are the main factors which prevent its application at commercial scale.

### 2.3.3.3 Ammonia fibre explosion (AFEX)

In AFEX process, the biomass is treated with liquid anhydrous ammonia at temperatures between 60 and 100°C with high pressure for variable time period. When the pressure is released it results in rapid expansion of ammonia gas that causes swelling and physical disruption of biomass and partial decrystallination of cellulose. Digestibility of biomass is increased after pretreatment (Galbe and Zacchi, 2007) which ultimately increases the sugar yield in enzymatic hydrolysis.

### 2.3.3.4 Wet oxidation

Wet oxidation is an oxidative pretreatment which employs air or oxygen as catalyst. The oxidation process is performed for 10-15 min at temperature ranges from 170 to 200°C and at pressures from 10 to 12 bar O₂(Olsson et al., 2005). Formation of acids from hydrolytic processes and oxidative reactions are the main reactions which occur in wet oxidation method. Furfural and hydroxy methyl furfural (HMF) production is lower in wet oxidation than steam explosion method and the addition of Na₂CO₃ has shown to decrease the formation of inhibitory compounds by maintaining the neutral to alkaline pH (Alvira et al., 2010). The cost of catalyst and oxygen are considered the main drawbacks for this method.

### 2.3.3.5 CO₂ explosion

Carbon dioxide explosion is also used as a pretreatment method for lignocellulosic biomass and it works on the basis of utilizing CO₂ as a supercritical fluid (fluid that is in a gaseous form but is compressed to a liquid like density at temperatures above its critical point). This supercritical treatment can effectively remove lignin which results in increased substrate digestibility (Schacht et al., 2008).

### 2.3.4 Biological pretreatments

Degradation of the lignocellulosic complex to liberate cellulose can be brought about with the help of microorganisms like white rot, brown rot and soft rot fungi, among which white rot fungi seem to be the most effective. Brown rot fungus attacks cellulose while white and soft rot fungi attack both cellulose and lignin (Prasad et al., 2007). This method is safe, saves energy and does not need chemicals. But, the low hydrolysis rate and sugar yield hinder its implementation (Sun and
Cheng, 2002; Talebnia et al., 2010). Biological delignification generally needs long incubation periods and both cellulose and lignin contents of the raw material may get reduced (Singh et al., 2008). Search is still going on to find efficient white rot fungi which are highly selective for lignin degradation resulting in holocellulose enrichment in the treated substrates (Saritha et al., 2012; Tiwari et al., 2013; Rana et al., 2013).

2.3.4.1 Lignocellulolytic enzymes

Many microorganisms including fungi and bacteria have been found to degrade cellulose and other plant cell wall fibres. Fungi contribute significantly to the decay of lignocellulosic residues by producing many different lignocellulolytic enzymes. Most fungal strains produce various enzymes in large amounts which are released into the environment and act synergistically in the breakdown of lignocellulosic biomass (Zhou and Ingram, 2000). Lignocellulolytic enzymes producing fungi are widespread, and include species from ascomycetes (e.g. *Aspergillus* sp., *Trichoderma* sp.) and basidiomycetes including white rot (e.g. *Phanerochaete chrysosporium*), brown-rot fungi (e.g. *Fomitopsis palustris*) and a few anaerobic species (e.g. *Orpinomyces* sp.) which degrade cellulose in gastrointestinal tracts of ruminant animals (Ljungdahl, 2008). These fungi degrade the biomass through complex mixtures of cellulases, hemicellulases and ligninases (Bayer et al., 1998; Weng et al., 2008). Cellulases and most hemicellulases belong to glycoside hydrolases (GH) enzyme group which have been identified (2500 GH) and classified into 132 families on the basis of their substrate specificity and occasionally on their molecular mechanism (Cantarel et al., 2009) (www.cazy.org).

**Fig. 2** Mode of action of cellulase enzyme complex on cellulose (www.brenda-enzymes.org)

2.3.4.1.1 Cellulases

Cellulases, comprising of many different enzymes hydrolyse the β-1,4 glycosidic bonds in cellulose by two different catalytic mechanisms, the retaining
and the inverting mechanisms. In both mechanisms, two catalytic carboxylate residues are involved and catalyze the reaction by acid-base catalysis. The fungal cellulose-degrading enzymes include endo-cleaving (endoglucanases) and exo-cleaving (cellobiohydrolases). Endoglucanases hydrolyze glycosidic bonds internally in cellulose chains whereas cellobiohydrolases act preferentially on chain ends (Fig. 2). The resulting products are mostly disaccharides known as cellobiose, and to a lesser extent, cello-oligosaccharides, which will be hydrolyzed further by a third group of enzymes called β-glucosidases (Kumar et al., 2008).

i) Endo-1,4-β-glucanases (E.C. 3.2.1.4, endocellulase)

Endoglucanases (EG) are also referred to as carboxymethylcellulases (CMCase), named after the (synthetic) substrate used to measure the enzyme activity. EG initiate cellulose breakdown by attacking the amorphous regions of the cellulose, making it more accessible to cellobiohydrolases by providing new free chain ends (Percival et al., 2006). Fungal EGs are generally monomers with no or low glycosylation and have an open binding cleft. They have optimum pH between 4 and 5 and temperature from 50 to 70°C.

ii) Cellobiohydrolases (E.C. 3.2.1.91, exocellulase)

Cellobiohydrolases (CBH) preferentially hydrolyze β-1,4 glucosidic bonds from chain ends, produces cellobiose as a main product. CBHs are similar to EGs in that they are monomers with no or low glycosylation with optimum pH between 4 and 5, but the optimum temperature is wider from 37 to 60°C. Cellobiose, the end product of CBHs, acts as a competitive inhibitor and can limit the ability of enzymes to degrade cellulose molecule (Sandgren et al., 2005; Baldrian and Valaskova, 2008).

iii) β-glucosidases (E.C. 3.2.1.21)

The β-glucosidases (BGL) hydrolyze β-1,4 glycosidic bonds in soluble cellobiose and cello dextrans to glucose by using the retaining mechanism, and are thus competitively inhibited by glucose. They have been isolated from many different fungal species including ascomycetes (Henrissat, 1991). BGLs have more variability among the cellulolytic enzymes due to their structure and localization; they have simple monomeric, dimeric and even trimeric structures and additionally most BGLs are glycosylated. Regarding localization, they can be grouped into three
different types which include intracellular, cell-wall associated and extracellular BGLs (Cai et al., 1999). The temperature optima range from 45 to 75°C.

2.3.4.1.2 Hemicellulases

Hemicelluloses are heterogeneous in nature, due to which several different enzymes are needed for their complete hydrolysis. Xylan is the most abundant component of hemicellulose and contributes over 70% of its structure. Xylanases are able to hydrolyze β-1,4 linkages in xylan and produce oligomers which can be further hydrolyzed into xylose by β-xylosidase. Additional enzymes such as β-mannanases, α-L-arabinanases are also needed depending on the composition of hemicelluloses (Shallom and Shoham, 2003). Similar to cellulose, most of the hemicellulases are glycoside hydrolases (GHs), although some hemicellulases belong to carbohydrate esterases (CEs) that hydrolyze ester linkages of acetate or ferulic acid side groups (Bourne and Henrissat, 2001). Aerobic fungi such as *Trichoderma* and *Aspergillus* secrete a wide variety of hemicellulases in high concentration and they work in a synergistic manner.

2.3.4.1.3 Ligninases

Fungi degrade lignin by secreting enzymes collectively termed as “ligninases”. These enzymes are expressed for a variety of purposes including defence and acquiring carbon and nitrogen. They are released into the environment through either excretion or lysis and mediate the functions like lignin degradation, humification and carbon mineralization (Sinsabaugh, 2010). These include two ligninolytic families - i) phenol oxidase (laccase) and ii) peroxidases [lignin peroxidase (LiP) and manganese peroxidase (MnP)] (Martinez et al., 2005). Interestingly, LiP is able to oxidize the non-phenolic part of lignin, but it was not detected in many ligninolytic fungi. In addition, it has been accepted that the oxidative ligninolytic enzymes are not able to penetrate the cell walls due to their size. Thus, prior to the enzymatic attack, low-molecular weight diffusible reactive oxidative compounds such as hydroxyl radicals have to initiate changes in the lignin structure (Tanaka et al., 1999).

i) Phenol oxidase (Laccase)

Laccases are widely produced by white rot basidiomycetes and soft rot ascomycetes fungi, but not by chitridiomycetes or zygomycetes (Bending and Read, 1997). Brown rot basidiomycetes produce laccases intracellularly and contribute to
the lignocellulose degradation upon cell lysis. Generally, the laccases of white rot fungi have lower pH optima (4.0-5.0) than the brown rot or coprophilic fungi (6.0-7.5). The enzymes of the former group are involved in lignin breakdown, while those of the latter contribute to humification by polymerizing the soluble phenols (Courty et al., 2009). Fungi are generally considered as the initiator of degradation of plant litter, especially lignin degradation, but most of the solubilised products of lignin degradation are metabolized by bacteria.

ii) Peroxidases

Some white rot and soft rot fungi produce extracellular peroxidases in addition to phenol oxidases. Lignin peroxidase and Mn peroxidase have Fe heme prosthetic groups. LiP can directly oxidize αC-βC bond between the phenyl propane units of lignin, but MnP indirectly attacks the lignin by creating diffusible Mn$^{3+}$. The activity of these enzymes is supported by aryl alcohol oxidases that generate H$_2$O$_2$ by oxidizing hydroxyls to carbonyls and reducing oxygen to hydrogen peroxide. Increase in the concentration of reactive oxygen species induces the peroxidase expression and increase of Mn availability induces MnP production (Sinsabaugh, 2010).

2.3.4.2 Application of lignocellulolytic microbes for biological pretreatment

In recent years, several researchers have reported the use of lignolytic fungi for biological pretreatment of different lignocellulosic biomass.

Kuhare et al. (2008) reported that fungal pretreatment of wheat straw for 10 days with a high lignin-degrading and low cellulose-degrading fungal isolate, RCK-1, resulted in a reduction in acid loading for hydrolysis, an increase in the release of fermentable sugars and a reduction in the concentration of fermentation inhibitors. Ethanol yield and volumetric productivity from RCK-1 treated wheat straw (0.48 g/g and 0.54 g/L/h, respectively) were higher than the untreated wheat straw (0.36 g/g and 0.30 g/L/h, respectively).

Biological pretreatment of rice straw with Dichomitussqualens increased the enzymatic digestibility to 58.1% of theoretical glucose yield and ethanol production yield and productivity were 54.2% of theoretical maximum and 0.39 g/L/h respectively after 24 hours (Bak et al., 2010).

The combined pretreatment of water hyacinth biomass with Echinodontiumtaxodii (10 days) and 0.25% H$_2$SO$_4$ was proved to be more effective
than sole acid pretreatment (Ma et al., 2010). The reducing sugar yield from enzymatic hydrolysis and ethanol yield (0.192 g/g of dry matter) from fermentation of co-treated water hyacinth increased 1.13-2.11 and 1.34 (0.146 g/g of dry matter) fold respectively than that from acid treated water hyacinth.

Salvachua et al. (2011) reported fungal pretreatment combined with a mild alkali treatment for enhanced saccharification. The glucose yield after 21 days of pretreatment with Poriasubvermispora and Irpexlactus reached 69% and 66% of available cellulose in the wheat straw, respectively, with an ethanol yield of 62% in both cases. Conversions from glucose to ethanol reached around 90% which showed that no inhibitors were generated during the pretreatment.

Gupta et al. (2011b) studied the biological delignification of lignocellulosic feedstocks (Prosopisjuliflora and Lantana camara) with Pycnoporuscinnabarinus, a white rot fungus, at different scales under solid state fermentation (SSF) and the fungal treated substrates were evaluated for their acid and enzymatic saccharification. The fungal fermentation at 10.0 g substrate level optimally delignified the P. juliflora by 11.89% and L. camara by 8.36%, and enriched their holocellulose content by 3.32 and 4.87%, respectively, after 15 days. The fungal delignification when scaled up from 10.0 g to 75.0, 200.0 and 500.0 g substrate levels, the fungus degraded about 7.69–10.08% lignin in P. juliflora and 6.89–7.31% in L. camara, and eventually enhanced the holocellulose content by 2.90–3.97% and 4.25–4.61%, respectively.

Liu et al. (2013) studied the fungal pretreatment of switch grass in solid state fermentation to improve the saccharification and simultaneously produce enzymes as co-products. Nearly 30% reduction in lignin content was obtained after a 36 days cultivation period without loss of cellulose and hemicellulose and the saccharification efficiency was about 50% greater than the untreated one.

A micromycete fungus, Myrotheciumroridum LG7 employed for delignification of paddy straw and Parthenium sp. for 7 days resulted in 6.98 and 5.8 mggds⁻¹ lignin recovery, respectively. Enzymatic hydrolysis of pretreated biomass significantly released high amounts of reducing sugars (455.8 mggds⁻¹ in Parthenium sp. and 509.6 mggds⁻¹ in paddy straw) as compared to raw biomass after 24 h (Tiwari et al., 2013). Rana et al. (2013) studied the biological pretreatment of Parthenium sp. with white rot fungus Trameteshirsuta for bioethanol production. The pretreated biomass yielded much higher recovery of sugars (485 mggds⁻¹) in 24
h of saccharification and illustrated the suitability of *Parthenium* as a feedstock for fuel ethanol production.

### 2.4 Genetic engineering of bioenergy crops for increased bioethanol production

In order to increase the structural carbohydrate content and reduce lignin levels in plant biomass, genetic engineering of crops may be considered a promising path that may reduce pretreatment severity, facilitate hydrolysis process and help in maximum sugar recovery. Additionally, the designed crops which express cellulose and hemicellulose degrading enzymes in cell wall decreases the overall cellulase enzyme load during saccharification step (Sticklen, 2008). Approaches have been adopted to increase stress tolerance, photosynthesis rate, grain yield and production of biomass conversion enzymes (Torney et al., 2007). These could be incorporated for the improvement of weedy crops in terms of cell wall composition, biomass weight and biomass conversion by enzyme expression (Chandel et al., 2011).

The research in lignin biosynthesis pathway may be helpful to reduce the lignin content by increasing the amount of cellulose for improved digestion and pulping efficiency (Reddy et al., 2005). Chen and Dixon (2007) studied the downregulation of lignin biosynthetic genes in alfalfa. It revealed an increment in fermentable sugars for improved ethanol production; this approach is advocated for other energy crops such as switch grass, *Miscanthus*, and poplar.

### 2.5 Enzymatic saccharification

The critical step for bioethanol production is saccharification where complex carbohydrates are converted to simple monomers. Enzymatic hydrolysis requires less energy and mild environment condition compared to acid hydrolysis (Ferreira et al., 2009). The optimum conditions for cellulase and xylanase have been reported as temperature of 40-50°C and 50°C, respectively and pH 4-5 (Park et al., 2002; Neves et al., 2007). Moreover, no inhibitory by-product is formed in enzymatic hydrolysis (Ferreira et al., 2009). However, enzymatic hydrolysis of different biomass is carried out by cellulase enzymes that are highly substrate specific (Taherzadeh and Karimi, 2007). The cellulase and hemicellulase enzymes cleave the bonds of cellulose and hemicelluloses, respectively. Cellulose contains glucan and hemicellulose contains different sugar units such as xylan, mannan, glucan, galactan and arabinan. Cellulase enzymes consist of endo and exoglucanase and β-glucosidases. Endoglucanase (endo
1, 4-D glucanhydrolase or E.C. 3.2.1.4) attacks the low crystallinity regions of the cellulose fiber, exoglucanase (1,4-β-D glucancellobiohydrolase or E.C. 3.2.1.91) removes the cellobiase units from the free chain ends and finally cellobiose units are hydrolysed to glucose by β-glucosidase (E.C. 3.2.1.21) (Taherzadeh and Karimi, 2007). Hemicellulolytic enzymes are more complex and a mixture of at least eight enzymes such as endo-1,4-β-D xylanases, exo-1,4-β-D xylocuronidases, α-L-arabinofuranosidases, endo-1,4-β-D mannanases, β-mannosidases (Jorgensen et al., 2003).

In saccharification process, cellulose is hydrolysed into glucose whereas hemicellulose gives rise to several pentoses and hexoses. Many fungi such as Trichoderma, Penicillium, Phanerochaete, Fusarium and Aspergillus have been reported to be useful for cellulase production. Among the various cellulolytic microbial strains Trichoderma is one of the most studied cellulase and hemicellulase producing fungi. It produces at least two cellobiohydrolases, five endoglucanases and three endoxylanases (Xu et al., 1998; Sandgren et al., 2001). However, Trichoderma lacks β-glucosidase activity that plays an efficient role in complete polymer conversion. On the other hand, Aspergillus is a very efficient β-glucosidase producer (Taherzadeh and Karimi, 2007). Chen et al. (2008) studied enzymatic hydrolysis of maize straw using cellulase from T.reesei ZU-02 and cellobiase from A.niger ZU-07. Combination of T. reesei ZU-02 cellulase and cellobiase from A. niger ZU-07 improved the hydrolysis yield to 81.2%.

Various factors influence the yield of monomer sugars during saccharification of lignocellulosic biomass like temperature, pH and mixing rate. Other factors that affect yield are substrate concentration, cellulase enzyme loading and surfactant addition (Sun and Cheng, 2002). High substrate loading may lead to substrate inhibition. Cellulase enzyme contributes to the major cost of the lignocellulosic ethanol technology (Banarjee et al., 2010). Therefore, an efficient pretreatment method has to be selected to decrease cellulose crystallinity and to remove lignin to the maximum extent, so that hydrolysis time as well as cellulase loading will be minimized (Eggman and Elander, 2005). Surfactants modify the cellulose surface by adsorbing lignin onto surfactant and thus the surfactant prevents the enzyme from unproductive binding with lignin and lowers enzyme loading (Eriksson et al., 2002). Addition of Tween 80 (5 g/L) improved hydrolysis yield by
7.5%, also it has been reported that addition of PEG increased the enzymatic conversion of soft lignocellulose from 42% to 78% at 16 h (Borjesson et al., 2007).

2.6 Fermentation for bioethanol production

Several microorganisms are used for fermentation of saccharified biomass, but industrial application of lignocelluloses for bioethanol production is impeded by the lack of ideal microorganisms which can ferment both pentose and hexose sugars efficiently (Talebnia et al., 2010). For commercially viable ethanol production method, an ideal microorganism should have a broad substrate utilization range, high ethanol yield and productivity, ability to withstand high concentrations of ethanol and high temperature and also tolerance to inhibitors present in the hydrolysate. Genetically modified microorganisms are used to achieve complete utilization of sugars in hydrolysate and better production benefits.

Separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) processes are usually involved in the fermentation of lignocellulosic hydrolysate. SSF is superior to conventional SHF process for ethanol production since it can improve ethanol yields by removing end product inhibition and eliminate the need of separate reactors. It is also cost effective but the optimum temperature conditions of enzyme for hydrolysis and fermentation differ and this poses some limitations (Bjerre et al., 1996; Hamelinck et al., 2005; Neves et al., 2007) that can be removed by using thermo-tolerant microorganisms like *Kluyveromyces marxianus* which has been developed to withstand the higher temperatures needed for enzymatic hydrolysis (Bjerre et al., 1996).

Apart from SHF or SSF, consolidated bioprocessing (CBP) and simultaneous saccharification and co-fermentation (SSCF) are the available alternatives (Cardona et al., 2009). Cellulase production, biomass hydrolysis and ethanol fermentation are all together carried out in a single reactor in CBP (Bjerre et al., 1996). Mono- or co-culture of microorganisms are generally used for the fermentation of cellulose directly to ethanol. CBP process does not require any capital investment for purchasing enzymes or its production (Hamelinck et al., 2005). In SSCF, the co-fermenting microorganisms need to be compatible for pH and temperature. A combination of *Saccharomyces cerevisiae* and *Candida shehatae* is suitable for the SSCF process (Neves et al., 2007). Sequential fermentation with two different microorganisms for better utilization of sugar has been reported using *S. cerevisiae*
during the first phase and *C. shehatae* in the second phase for hexose and pentose utilization, respectively, but ethanol yields achieved were not high (Sanchez and Cardona, 2008).

Some wild and native microorganisms used in fermentation are *S. cerevisiae*, *Escherichia coli*, *Zymomonas mobilis*, *C. shehatae*, *Pichiastipitis*, *C. brassicae*, *Mucorindicus* etc. (Bjerre et al., 1996; Sanchez and Cardona, 2008; Talebnia et al., 2010). Among all, the best known yeast and bacteria employed in ethanol production from hexoses are *S. cerevisiae* and *Z. mobilis*. The fermentation of xylose is also essential for the bioconversion of lignocellulose to fuels, but *S. cerevisiae* cannot utilize C-5 sugar (xylose) of the hydrolysate (Talebnia et al., 2010). Native microorganisms such as *Pichia* and *Candida* can utilize xylose but their ethanol production rate is at least fivefold lower than that of *S. cerevisiae* (Xu et al., 1998).

Although the use of co-cultures of both hexose and pentose fermenting yeasts is desired, different oxygen requirements especially during the exponential growth phase of organisms and different metabolic pathways for carbon utilization and energy production pose operational problems during fermentation. Hence researchers have engineered xylose metabolism in hexose fermenting yeasts, especially *S. cerevisiae*.

Genetic engineering has been employed for the improvement of various aspects of fermentation from higher yield and broad substrate utilization to increased recovery rates. Many improvements have been made for the fermentation of xylose and arabinose to ethanol and other products such as lactic acid. However, bioconversion of pentoses to ethanol still presents a considerable economic and technical challenge (Hahn-Hägerdal et al., 2001). Glucose transporters mediate xylose uptake, but this competes with glucose uptake; this indicates that they have common transport components. No transporter specific for xylose has yet been identified. Glucose transporters exhibit lower affinity for xylose than for glucose; therefore glucose and xylose consumed simultaneously only under glucose limited conditions (Meinander and Hahn-Hägerdal, 1997). *P. stipitis* produces relatively little xylitol than the other pentose fermenting yeasts. *S. cerevisiae* engineered with xylose fermenting genes of pentose phosphate pathway such as XYL1, XYL2 and XL3 (xylose reductase, xylitol dehydrogenase and xylokinase, respectively) from *Pichia* tend to produce xylitol (Hallborn et al., 1994). The xylitol production can be reduced by increasing the expression of XYL2 relative to XYL1 (Jin et al., 2003) to
enhance the ethanol production. *S. cerevisiae* ATCC 26603 (Moniruzzaman, 1995), *P. stipitis* BCC15191 (Buaban et al., 2010), *C. shehatae* NCL-3501 (Abbi et al., 1996), recombinant *E. coli* KO11 (Nigam, 2001) have been developed through genetic engineering for ethanol production.

The development of efficient microorganisms for fermentation can follow three ways: (a) making *C. shehatae, P. stipitis* and recombinant *E. coli* more resistant to inhibitors (b) genetic engineering of *S. cerevisiae* or *Z. mobilis* for xylose fermentation (c) metagenomics of natural genes to develop an efficient fermentation process. For sustainable generation of biofuels, exploring modern genetic engineering tools to produce tailor-made perennial plants and trees with increased amounts of biomass and to develop microbes which ferment both hexose and pentose sugars is an unavoidable necessity (Harris et al., 2009).

In conclusion, it can be inferred that the weedy biomass Parthenium is difficult to control because of its wide adaptability, regeneration and invasion capacity along with health hazards to human and livestock and therefore, needs to be managed. Since this weedy biomass has considerable amount of holocellulose (about 60%) it can be used as a feedstock for ethanol production at farm level. Bioethanol production from Parthenium could be an attractive alternative for the management of these residues. Importantly, it does not interfere with the availability of land and food security. Therefore, in the present study, the Parthenium biomass was employed as a substrate for ethanol production by chemical/biological pretreatment and enzymatic saccharification.
3. MATERIALS AND METHODS

3.1 Collection of samples and isolation of lignocellulolytic fungi

Various plant samples which includes leaf litter, barks and rotten fruits were collected from Pachmarhi hill station (situated at a height of 1100 m (22.46°N 78.43°E) in a valley of the Satpura range in Hosangabad district in Madhya Pradesh), Almora (located in the elevation of 1651 m on a ridge of the Kumaon Hills (29.62°N 79.67°E) of the Himalayan range) and Cauvery river basin (11.37°N 78.20°E) near Namakkal district in Tamil Nadu. Isolation of lignocellulolytic fungi was done by inoculating the samples on Reese’s mineral medium (Reese and Mandels, 1963) with 1% alkali lignin (Sigma) / carboxymethyl cellulose (CMC) as carbon source.

Reese’s mineral medium (RMM):

\[
\begin{align*}
KH_2PO_4 & \quad - \quad 2g \\
(NH_4)_2SO_4 & \quad - \quad 1.4g \\
KNO_3 & \quad - \quad 1.4g \\
MgSO_4.7H_2O & \quad - \quad 0.3g \\
CaCl_2.2H_2O & \quad - \quad 0.3g \\
FeSO_4.7H_2O & \quad - \quad 5mg \\
MnSO_4.2H_2O & \quad - \quad 1.6mg \\
ZnSO_4.7H_2O & \quad - \quad 1.4mg \\
CoCl_2.6H_2O & \quad - \quad 2mg \\
Agar & \quad - \quad 20g \\
\text{Distilled water} & \quad - \quad 1000 ml
\end{align*}
\]

The pH was adjusted to 5.0 with 1 N HCl or NaOH solution and stored at 4°C. The cultures were further purified and subcultured on potato dextrose agar (PDA) and stored at 4°C for screening of lignolytic and cellulolytic enzymes.
3.2 Qualitative screening of fungal isolates for lignocellulolytic enzymes

3.2.1 Cellulolytic activity

3.2.1.1 Zone of hydrolysis on CMC agar

The cellulolytic activity of fungal isolates was performed in petridish on Reese’s mineral medium (RMM) supplemented with 1% carboxymethyl cellulose (CMC) and incubated for 3 days at 30°C. For visualization of hydrolysis zone, the plates were flooded with congo red solution (1mg/ml) for 15 min. This solution was then drained off and the plates were destained twice by 1 M NaCl solution for 15 min (Teather and Wood, 1982). The yellow zone around the fungal growth indicated presence of cellulolytic activity.

3.2.1.2. Growth on Reese’s medium with acid swollen cellulose as carbon source

The cellulolytic ability of isolates was assayed on the basis of their ability to grow on Reese’s mineral medium with acid swollen cellulose as sole carbon source (Rautela and Cowling, 1966).

The swollen cellulose was prepared by dissolving 10 g of microcrystalline cellulose powder in 85% o-phosphoric acid for 2 h at 4°C and washed with cold distilled water followed by addition of Na₂CO₃ (1% w/v) solution and then with distilled water until pH became neutral. The isolates were point inoculated on the plates containing the above mentioned media. The growth and zone of clearance indicated the cellulolytic enzyme production.

3.2.2. Xylanolytic activity

The isolates were screened for xylanolytic activity by their growth on RMM with 0.1% (w/v) xylan as sole carbon source (Rautela and Cowling, 1966). The substrate was prepared according to Bailey et al. (1992) by dissolving 1 g of oat spelt xylan in 1 ml of 1 N NaOH by stirring on magnetic stirrer for 10 min and the pH was brought up to 6.0 using sodium phosphate buffer (50 mM, pH 6.0). This solution was added to RMM and the final volume made up to 1000 ml. Growth on the inoculated plates indicated capability of the isolated microbe to produce xylanase enzyme.

3.2.3 Lignolytic activity

3.2.3.1. Chromogenic test on Basal agar medium (Thorn et al., 1996)
For lignolytic activity, agar plug (6mm diameter) of the one week old fungal isolates were inoculated on Basal agar medium containing lignin to encourage the selection of lignolytic fungi and guaiacol which acts as a chromogenic substrate to indicate the presence of lignin-modifying enzymes such as laccase or lignin peroxidase.

**Basal agar medium:**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{KH}_2\text{PO}_4$</td>
<td>0.5g</td>
</tr>
<tr>
<td>$\text{MgSO}_4.7\text{H}_2\text{O}$</td>
<td>0.2g</td>
</tr>
<tr>
<td>$\text{NH}_4\text{NO}_3$</td>
<td>0.1g</td>
</tr>
<tr>
<td>$\text{KCl}$</td>
<td>0.1g</td>
</tr>
<tr>
<td>$\text{FeSO}_4.7\text{H}_2\text{O}$</td>
<td>0.02g</td>
</tr>
<tr>
<td>$\text{Ca(NO}_3)_2.4\text{H}_2\text{O}$</td>
<td>0.05g</td>
</tr>
<tr>
<td>Malt Extract</td>
<td>2g</td>
</tr>
<tr>
<td>Agar</td>
<td>15g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Basal medium was autoclaved and cooled approximately to 55°C when the following were added aseptically: 5 ml of 1 M KOH; 0.4 ml of guaiacol; 1 gram of alkali lignin (Sigma) which was suspended and partially dissolved in 10 ml of dioxane. The medium was stirred and poured into the sterile petridishes. The formation of bright red zone beneath the colonies indicates the production of laccase or lignin peroxidase.

### 3.3 Quantitative screening of fungal isolates for lignocellulolytic enzymes

For quantitative estimation of extracellular lignocellulolytic enzymes, the fungal isolates were grown under submerged fermentation for extraction of extracellular enzymes.

#### 3.3.1 Submerged fermentation for the production of enzyme

The submerged fermentation was carried out and culture filtrate of fungal isolates was collected for quantitative assay of lignocellulolytic enzymes. For this, 0.1 g of air dried *Parthenium* substrate was added to the 10 ml of RMM (1%) in tubes
followed by autoclaving at 121°C for 15 min. The tubes were inoculated with an agar plug (6 mm diameter) of one week grown fungal isolates and incubated at 30°C for 7 days. The supernatant was filtered through filter paper and the filtrate was used for the estimation of extracellular lignocellulolytic enzyme assay.

3.3.2 Cellulolytic enzymes

Cellulolytic enzymes which include, CMCase (Endoglucanase), FPase, β-D-glucosidase (Cellobiase) and xylanolyic enzyme namely xylanase were estimated by using the culture filtrate with appropriate substrates.

3.3.2.1 Carboxymethyl cellulase: (β-1,4Endoglucanase; E.C. 3.2.1.4)

Carboxymethyl cellulase (CMCase) activity was assayed using the substrate CMC as described by Ghose (1987). For this, 2% CMC solution was prepared in 50 mM sodium citrate buffer (pH 4.8) and stored at 4°C until further use. The reaction mixture contains 500 µl of 2% CMC and 500 µl of enzyme filtrate with appropriate dilution which was incubated at 50°C for 30 min along with substrate blank which contains no enzyme filtrate. After incubation, 3 ml of dinitro salicylic acid (DNSA) reagent was added to stop the reaction and kept in boiling water bath for 16 min and cooled (Miller, 1959). The amount of released glucose was measured spectrophotometrically at 575 nm.

3.3.2.2 Cellobiohydrolase or FPase: (β-1,4 Exoglucanase; E.C. 3.2.1.91)

FPase activity was estimated according to Ghose (1987) method by using filter paper as a substrate. 50 mg of filter paper pieces were weighed to which 500µl of sodium citrate buffer and enzyme filtrate with dilution were added to make the reaction mixture to 1 ml. The reaction mixture was incubated 50°C for 1 hour along with substrate blank. Three ml of DNSA reagent was added to stop the reaction and kept in boiling water bath for 16 min and cooled. The amount of glucose released was measured at 575 nm using spectrophotometer.

3.3.2.3 Cellobiase or pNPGase: (β-D-glucosidase; E.C. 3.2.1.21)

Cellobiase enzyme activity was assayed using β-D-glucopyranoside as a substrate (Wood and Bhat, 1988). The substrate was prepared by adding 0.015 g of p-nitrophenyl-β-D-glucopyranoside (4 mM) in 10 ml of sodium citrate buffer (pH 4.8). The reaction mixture was incubated at 50°C for 30 min along with substrate
blank and the reaction was stopped by adding glycine buffer (pH 10.8). The amount of p-nitrophenol released was measured at 430 nm.

3.3.2.4 Xylanase: (E.C. 3.2.1.8)

Xylanase activity was assayed by using xylan as substrate according to Ghose and Bisaria (1987). For this, 1% xylan was prepared in 10 ml of sodium citrate buffer (pH 4.8) by dissolving 0.1 g of xylan in 1 ml of 1 M NaOH on magnetic stirrer. The pH was adjusted to 4.8 with sodium citrate buffer and the final volume made up to 10 ml. The enzymatic reaction mixture containing 500µl of substrate and 500µl of culture filtrate with appropriate dilution was incubated at 50°C for 30 min along with substrate blank. After incubation, 3 ml of DNSA reagent was added to stop the reaction and kept at boiling water bath for 16 min and cooled. The amount of xylose released was measured at 575 nm using spectrophotometer. One unit of (IU) activity was defined as the amount of enzyme capable of liberating 1µ mole of end product under assay conditions.

Dinitro salicylic acid (DNSA) reagent:

Solution A: (1% NaOH)

NaOH - 10g  
Distilled water - 1000 ml

Solution B:

Dinitrosalycilic acid - 10 g  
(Crystal) phenol - 2 g  
Na-K tartrate - 0.2 g  
Solution A - 1000 ml

Solution A and Solution B was kept at 4°C until further use.

Solution C: (5% Sodium sulphite)

Sodium sulphite - 5 g  
Distilled water - 100 ml

Preparation of working solution:

Solution B - 99 ml  
Solution C - 1 ml

50mM Sodium Citrate buffer: (pH 4.8)

Solution A:
Citric acid monohydrate - 10.55 g
Distilled water - 1000 ml

Solution B:
Tri-sodium citrate - 14.7 g
Distilled water - 1000 ml

Buffer:
Solution A (440 ml) and solution B (560 ml) were mixed together to prepare citrate buffer of pH 4.8 and stored at 4°C.

3.3.3 Lignolytic enzymes
Lignolytic enzymes namely laccase and lignin peroxidase were assayed by using ABTS and Veratyl alcohol as substrates.

3.3.3.1 Laccase: (E.C. 1.10.3.2)
Laccase activity was assayed by using ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) as a substrate as described by Childs and Bardsley (1975). 5 mM ABTS was prepared in sodium citrate buffer (pH 4.8) for the enzyme estimation. In the enzymatic reaction mixture of 500µl of culture filtrate and 500µl of sodium citrate buffer, 100µl of ABTS was added and the absorbance was measured through time scanning for 3 min with time intervals of 30 sec at 420 nm in spectrophotometer. The reference cuvette does not have ABTS substrate. The enzyme activity was expressed in units (IU) defined as the amount of enzyme oxidizing of ABTS per minute ($\varepsilon_{420} = 36,000 \text{ M}^{-1}\text{cm}^{-1}$).

3.3.3.2 Lignin peroxidase: (E.C. 1.11.1.14)
Lignin peroxidase activity was estimated according to Tien and Kirk (1984). The reaction mixture was containing 500µl 100 mM tartrate buffer (pH 3.0), 500µl culture filtrate with appropriate dilution and 150µl veratyl alcohol (2mM), 150µl $\text{H}_2\text{O}_2$(10mM). In the reference cuvette $\text{H}_2\text{O}_2$ was absent. The veratyl alcohol is oxidised to varataraldehyde by the action of $\text{H}_2\text{O}_2$ and it was measured at the wavelength of 310 nm ($\varepsilon_{420} = 9,300 \text{ M}^{-1}\text{cm}^{-1}$) by time scanning for 3 min with time intervals of 30 sec. One unit (IU) of enzyme activity defined as the amount of enzyme oxidizing the 1µ mole of veratyl alcohol per minute.

3.3.4 Estimation of total soluble proteins
Total amount of protein present in the culture filtrate was determined by Bradford method (1976). One ml of culture filtrate was taken in test tube to which 5 ml of Bradford reagent was added. The mixture was vortexed and kept at stable condition for 10 min for the colour development. The amount of protein was measured at 660 nm and the amount of protein was determined by plotting the absorbance against bovine serum albumin (BSA) standard curve. The reagent was prepared by dissolving 133 mg of Coomassie brilliant blue G-250 in 50 ml of 95% ethanol to which 100 ml of 85% phosphoric acid was added and the volume made up to 1000 ml with distilled water. The solution was filtered through whatman no.1 filter paper and stored at 4°C.

3.3.5 Estimation of total phenol

Total amount of phenol present in the culture filtrate was estimated by method of Bray and Thorpe (1954). To the culture filtrate (0.5 ml), 0.5 ml of 1 N Folin ciocalteau reagent (prepared by adding equal volume of distilled water with 2 N Folin ciocalteau) and 1 ml of 20% (w/v) Na₂CO₃ were added and kept in boiling water bath for 1 min and cooled. 3 ml of distilled water was added to make the volume up to 5 ml and measured spectrophotometrically at 660 nm. The amount of phenol was determined by plotting the absorbance against standard curve of tannic acid.

3.3.6 Molecular characterization of fungal isolates

On the basis of qualitative and quantitative determination of lignocellulolytic enzymes, the most promising isolates were selected for their molecular characterization and identification. DNA was extracted from selected isolates by using ZR Fungal/Bacterial DNA kit (Zymo Research, California, USA). The rDNA gene cluster (ITS-1, 5.8S rDNA and ITS-2) was amplified by using the universal primers pITS-1 (5’-TCCGTAGGGTGAACCTGCGG-3’) and pITS-4 (5’-TCCTCCGCTTATTGATATGC-3’) (White et al., 1990).

The PCR program was as follows: initial incubation at 94°C for 5 min followed by 35 cycles (94°C for 50s, 55°C for 1 min and 72°C for 90s) and final extension at 72°C for 10 min using thermal cycler (BioRad). Amplified fragments of approx. 650 bp was sequenced at Eurofins Genomics India Pvt. Ltd, Bangalore. The partial sequences of the isolates were compared with sequences available by the blast

**PCR reaction:**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Components</th>
<th>Stock solution</th>
<th>Working solution</th>
<th>Volume/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Buffer</td>
<td>10 X</td>
<td>1 X</td>
<td>10 µl</td>
</tr>
<tr>
<td>2.</td>
<td>dNTP</td>
<td>10 mM</td>
<td>0.2 mM</td>
<td>2 µl</td>
</tr>
<tr>
<td>3.</td>
<td>Primer F</td>
<td>10 µM</td>
<td>0.2 µM</td>
<td>2 µl</td>
</tr>
<tr>
<td>4.</td>
<td>Primer R</td>
<td>10 µM</td>
<td>0.2 µM</td>
<td>2 µl</td>
</tr>
<tr>
<td>5.</td>
<td>Taq polymerase</td>
<td>3 U/µl</td>
<td>1.5 U</td>
<td>2 µl</td>
</tr>
<tr>
<td>6.</td>
<td>DNA</td>
<td></td>
<td></td>
<td>6-7 µl</td>
</tr>
<tr>
<td>7.</td>
<td>Nuclease free water</td>
<td></td>
<td></td>
<td>75 µl</td>
</tr>
</tbody>
</table>

3.4 Pretreatment of Parthenium by biological and chemical methods

3.4.1 Biological pretreatment of Parthenium

Five grams of air dried Parthenium was transferred to 500 ml conical flask mixed with 25 ml of RMM in the ratio of 1:5 and inoculated with 5 agar plugs (6 mm diameter) of one week old fungal isolate (*Marasmiellus palmivorus* PK-27). The inoculated samples were kept in static condition at 30°C for 7 days. After incubation, the samples were washed with 0.1 N NaOH to remove the mycelial biomass and to remove the lignin from the samples. Further, the samples were washed with distilled water until the neutral pH and the extracts were kept at 4°C until further analysis. The absorbance of alkali washed extracts were measured at 205 nm for determining the extent of delignification (Sluiter et al., 2008) and to estimate the recovery of lignin as acid perceptible polymerised lignin (APPL). Biologically pretreated samples were used for the enzymatic hydrolysis (saccharification).

3.4.2 Chemical pretreatments of Parthenium

Five grams of Parthenium biomass sample was taken with 1% (v/v) dilute H₂SO₄ with the substrate loading of 1:5 ratio in a screw capped flask which was loosely closed and kept at 121°C for 20 min. For alkali treatment, 5 g of dried sample was mixed with 1% (w/v) NaOH in a flask and kept at static condition at room temperature for 1 hour. The chemically treated samples were filtered through
muslin cloth and washed with tap water for acid treated and with distilled water for alkali treated samples until neutral pH and stored at 4°C. The extracts were used to measure the absorbance at 205 nm. The pH of the alkali wash was adjusted to 2 with conc. H₂SO₄ to precipitate the lignin and to estimate APPL yield. Acid and alkali pretreated samples were further used for enzymatic hydrolysis.

3.5 Estimation of extent of colonization by *M. palmivorus* PK-27 on *Parthenium*

The extent of colonization of fungus was estimated in terms of N-acetyl glucosamine according to Aidoo et al. (1981). The fungal cell wall contains chitin which is converted into N-acetyl glucosamine and estimated spectrophotometrically.

After biological pretreatment of Parthenium with *M. palmivorus* PK-27, 0.1 g of pretreated sample was taken in a screw capped tube and mixed with 5 ml of 2 N HCl which was kept in boiling water bath for 2 hours and cooled. Then the volume was made up to 5 ml by using 2 N HCl. A drop of phenolphthalein was added and titrated with 1 N NaOH until the solution turned pink colour. The solution was again titrated with drop wise addition of 1% potassium hydrogen sulphate (KHSO₄) until the colour just disappeared. From this, 0.5 ml was taken in to test tube and 0.5 ml of distilled water was added to make the volume to 1 ml also 1 ml of 5% (w/v) sodium nitrite and 1 ml of 5% (w/v) KHSO₄ and the tubes were incubated at room temperature for 15 mins. One ml of 12.5% (w/v) ammonium sulphamate was added and vortexed for 5 min. One ml of 0.5% (w/v) MBTH (3-methyl-2-benothiazole hydrazone hydrochloride) was added and kept in boiling water bath for 5 min. After cooling, 1 ml of 0.83% (w/v) ferric chloride solution was added to the tubes, mixed well and kept for 30 min for colour development. The absorbance of the samples was measured at 650 nm against distilled water as reference using spectrophotometer.

3.6 Analysis of structural and chemical changes of pretreated *Parthenium*

The biological and chemical pretreated *Parthenium* samples along with raw sample were used for analysis of changes in the biomass structure and its chemical composition through scanning electron microscopy (SEM), X-ray diffraction (XRD) and Fourier transform infrared (FTIR) analysis.

3.6.1 Scanning electron microscopy (SEM) analysis

The pretreated and raw samples were fixed in 2.5% glutaraldehyde for overnight and washed with 0.1 M phosphoric acid. The washed samples were gradually dehydrated with acetone by gradually increasing the concentration up to
100%. Finally, the samples were coated with gold/palladium (Au/Pd) and imaging was done at different magnifications between 500X to 5000X with ZEISS (EVOMA 10) scanning electron microscope at 20 kV/ EHT and 10 Pa.

3.6.2 X-ray diffraction (XRD) analysis

The raw and pretreated samples were ground into particle size of less than 1 mm and scanned at a speed of 1°/min in the range (2θ) between 10° and 35° by using Cu-ka radiation at 40 kV and 25 mA (PW 1729 Philips X-ray generator with PW 1710 diffractometer). The biomass crystallinity index (CrI) was determined according to the method described by Segal et al. (1959).

\[
\text{CrI} = \left( \frac{I_{002} - I_{\text{amorphous}}}{I_{002}} \right) \times 100
\]

in which, \(I_{002}\) is the intensity for the crystalline portion of biomass (i.e., cellulose) at about \(2\theta = 22.5\) and \(I_{\text{amorphous}}\) is the peak for the amorphous portion (i.e., cellulose, hemicelluloses and lignin) at about \(2\theta = 16.2\).

3.6.3 Fourier transform infrared (FTIR) analysis

Pretreated and raw samples were finely ground and pressed uniformly into the disc and analyzed using Attenuated Reflectance – Fourier transform infrared (ATR - FTIR) spectroscopy (ALPHA-E, Bruker) with the resolution of 1 cm\(^{-1}\) over the wavelength range between 4000 cm\(^{-1}\) to 600 cm\(^{-1}\).

3.7 Enzymatic saccharification of pretreated Parthenium

Saccharification of the pretreated samples was carried out according to the NREL LAP-009 (Brown and Torget, 1996) with slight modifications. One gram of fresh samples of pretreated Parthenium was taken into 50 ml screw capped bottle to which 9.5 ml of 50 mM sodium citrate buffer (pH 4.8), 0.5 ml of cellulolytic enzyme complex, Accellerase®1500 (endoglucanase activity; 2200 – 2800 CMC U/g, β-glucosidase activity; 525 – 775 pNPG U/g) and 100 µl of 2 % Sodium azide (to prevent any microbial contamination during saccharification) was added and incubated in shaking water bath at 50°C for 48 hours. The samples were taken at different intervals (0, 16, 24, 40 and 48 hours), centrifuged at 10000 rpm for 5 min. The supernatant was analyzed for total reducing sugars by DNSA method (Miller, 1959) and expressed as.

\[
\text{Saccharification (\%)} = \frac{\text{Reducing sugars released (mg)} \times 0.9}{\text{Carbohydrate content in pretreated biomass}} \times 100
\]
3.8 Compositional analysis of Parthenium sp.

The raw and pretreated Parthenium sp. samples were analyzed for cellulose, hemicelluloses and lignin, moisture and ash contents.

3.8.1 Preparation of sample for chemical analysis

The sample was prepared for compositional analysis according to TAPPI (T 264) method. The biomass extractives are materials soluble in neutral solvents and are not generally considered as part of the lignocellulosic substrates. Ethanol-benzene is used to extract waxes, fats, some resins and possibly some portions of gums.

The sample was placed in the extraction thimble which was placed in position in the Soxhlet apparatus. Small amount of cotton was placed in the top of the thimble to prevent any loss of the sample and extracted with 200 ml of ethanol-benzene solvent (prepared by mixing one volume of ethanol with two volumes of the benzene) for 8 h. After extraction, the sample was transferred to Buchner funnel and the excess solvent was removed with suction. The thimble and sample was washed with ethanol to remove the benzene and returned to extraction thimble for extraction with 95% ethanol for 4 h or longer if necessary, until the alcohol siphons over colourless. The sample was again transferred to a Buchner funnel to remove the excess solvent with suction and washed with distilled water to remove the ethanol. Then the sample was transferred to 1000 ml flask to which 500 ml of distilled water was added and boiled for 1 h in the hot water bath. After extraction, the sample was transferred to Buchner funnel to remove the excess water with suction and the sample was allowed to air dry thoroughly. The dried sample was mixed and stored in an air tight container.

3.8.2 Cellulose estimation

The cellulose content was estimated by the method described by Updegraff (1969). Acetic/nitric reagent was prepared by mixing 450 ml of 80% acetic acid and 45 ml of concentrated nitric acid and inverted for 4 times for proper mixing. The samples (50 mg) were taken into a 15 ml centrifuge tube and 3 ml of acetic nitric reagent was added in such a manner that initially 1.5 ml was added, mixed well on vortex mixer, and then added the remaining 1.5 ml of reagent and remixed. The tubes are loosely caped and placed in a boiling water bath for 30 min and centrifuged at 2000 rpm for 5 min. The supernatant was removed and 10 ml of distilled water was added in a manner similar to addition of acetic nitric reagent, centrifuged at
2000 rpm for 5 min and the supernatant was decanted. 10 ml of 67% H$_2$SO$_4$ (v/v) was added in a manner similar to addition of acetic nitric reagent and kept at room temperature for 1 h. Five ml of sample was diluted to 50 ml by adding distilled water and 1 ml of this dilution was mixed with 4 ml of distilled water in a wide mouth glass tube. 10 ml of cold anthrone reagent was added gradually that is 2.5 ml of reagent was added and vortex mixed and again 2.5 ml of reagent and vortex mixed and finally the remaining 5 ml of reagent was added and vortex mixed. The tubes were loosely caped and kept in a boiling water bath for 16 min and cooled in a ice bath for 2 min and kept at room temperature for 5 min. The absorbance was measured at 620 nm using spectrophotometer. The amount of cellulose was calculated by using the standard curve for cellulose. Anthrone reagent was prepared freshly on the day of analysis by dissolving 0.5 g of anthrone in 250 ml of concentrated H$_2$SO$_4$ and chilled for 2 h in refrigerator prior to use.

The standard curve was prepared by dissolving 50 mg of cellulose in 10 ml of 67% H$_2$SO$_4$ and diluted to 500 ml with distilled water to contain 100 µg cellulose / ml. From this dilution standard was prepared with different concentrations (50 - 350 µg / ml) and the final volume made up to 5 ml.

### 3.8.2 Pentosans estimation

Pentosans present in the sample were estimated according to TAPPI (T 223) method. Pentosans are transformed in boiling with HCl (3.85 N) to furfural, which is collected in the distillate and the pentose sugars were determined colorimetrically with orcinol - ferric chloride reagent.

The sample (0.5 g) was placed in a boiling flask and 20 g of NaCl, 100 ml of HCl (3.85 N) and a few boiling stones were added. The flask was connected to the distillation apparatus and the acid level in the flask was marked. To the separatory funnel 250 ml of 3.85 N HCl was added. The acid was distilled at a uniform rate and collected in a 250 ml volumetric flask immersed in an ice bath. During distillation, the volume of 100 ml in boiling flask was maintained by the addition of HCl drop wise from the separatory funnel. The distillation was continued for 3 h, in this time 95 ± 2 ml of distillate was collected. The temperature of the distillate was brought down to 20°C and HCl (3.85 N) was added to the 250 ml mark and mixed thoroughly. Orcinol reagent was prepared by dissolving 0.4 g of orcinol and 0.5 g of ferric chloride in 1000 ml of HCl (11 N). Five ml of distillate was pipetted in a 50 ml
volumetric flask to which 25 ml of orcinol reagent was added and placed in a water bath at 25°C. After 1 h, ethanol was added up to the 50 ml mark, mixed and returned to the water bath for another 1 h. The absorbance was measured with a spectrophotometer at 630 nm. The concentration of pentosans was calculated as:

$$\text{Pentosans (\%) = \frac{\text{Xylan in test specimen (mg)}}{\text{Oven dry weight of test specimen (g)} \times 10}}$$

$$\text{Xylan (mg) = Xylose (mg) \times 0.88}$$

Xylose concentration was derived from calibration graph which was prepared by boiling xylose range from 10 to 100 mg (10, 20, 40, 60, 80, 100 mg) with HCl (3.85 N) in flask and the distillation and colour development processes were done as mentioned above. The blank contain 5 ml of HCl (3.85 N) instead of distillate as the reference solution.

### 3.8.3 Lignin estimation

The lignin content of the samples was estimated according to TAPPI (T 222) method. In this method of determination, lignin (also known as “Klason lignin”) is defined as the constituent insoluble in 72% sulphuric acid. One gram of sample was placed in a 100 ml beaker to which 15 ml of 72% sulphuric acid was added gradually in small increments while stirring the material in vortex and kept in a water bath at 20°C for 2 h. About 400 ml of water was added to a flask and the material was transferred from beaker to the flask and diluted to 3% concentration of sulphuric acid, to a total volume of 575 ml and boiled for 4 h, by maintaining the constant volume through refluxing. The insoluble material was allowed to settle by keeping the flask for overnight. The supernatant solution was decanted through a filtering crucible by vacuum filtration. The crucible was dried in an oven to a constant weight.

### 3.8.4 Moisture and Ash determination

The moisture and ash content was determined by the method described by TAPPI (T 211). For moisture determination, sample (1 g) was weighed and dried for 2 h at 105°C and weighed and returned to oven for 1 h. The weighing and drying was repeated until constant weight. In ash determination, the sample (1 g) was transferred to the crucible and placed in a furnace and the temperature of the furnace
was raised to 525°C for 5 h. When the sample is completely combusted, the crucible was removed and weighed to the nearest 0.1 mg.

3.9 Optimization of saccharification by Response Surface Methodology (RSM)

Response surface methodology (RSM), a collection of statistical and mathematical techniques, is normally used for modelling and analyzing problems in which several variables influencing the response of interest and the aim is to optimize the response (Ferreira et al., 2009). The sugar yield of enzymatic hydrolysis in terms of saccharification efficiency was taken as the response influenced by many potential variables. In this study, a central composite rotatable design was employed to determine the effects of independent variables on response and to optimize the enzymatic hydrolysis. Thirty runs of experiment were formed by Design Expert® 8.0.7.1 (Stat-Ease, Inc., Minneapolis, USA) with six replications at the central point and one replicate at axial and factorial point in a single block. This software is widely used for RSM in bioprocess optimization because of its convenience in statistical analysis and experimental design (Ferreira et al., 2009). The variables include temperature (A), pH (B), enzyme loading (C) and substrate loading (D).

Using Design Expert® 8.0.7.1, all the statistical and mathematical analysis of the results was done to evaluate the effects of variables and their interactions. Three-dimensional surface plots were drawn to illustrate the effects of independent variables on response and a quadratic polynomial equation was proposed to describe the mathematical relationship between the variables and the response. All experiments were conducted in triplicate and the mean values of sachharification efficiency from enzymatic hydrolysis were taken as a response. The significance of the model was evaluated by determination of R² and adjusted R² coefficient. The validation of the model optimum value of selected variables was obtained by solving the regression equation using Design Expert® 8.0.7.1. The predicted optimum response was confirmed by the experiment using the selected optimum values of the four variables. The saccharified material was prepared by taking alkali pretreated Parthenium biomass in a container using the optimized substrate and enzyme loading rate at particular temperature and pH.
3.10 Fermentation of sugar hydrolysate from *Parthenium*

The sugar hydrolysate of alkali pretreated substrate was obtained from enzymatic saccharification which was performed under the optimized process conditions which include temperature (50°C), pH (4.53), enzyme loading (0.8 ml) and substrate loading (0.24 g) in shaking water bath for 48 h. The hydrolysates were fermented by using *Saccharomyces cerevisiae* LN1 and *Pichia stipitis* NCIM 3498.

*Saccharomyces cerevisiae* LN1 was obtained from the culture collection by Division of Microbiology, Indian Agricultural Research Institute, New Delhi, India and *P. stipitis* NCIM 3498 was procured from National Collection of Industrial Microorganism (NCIM), National Chemical Laboratory (NCL), Pune, India. Both yeasts were maintained in Malt extract - Glucose - Yeast extract - Peptone (MGYP) medium.

MGYP composition:

- Malt extract - 3 g
- Glucose - 10 g
- Yeast extract - 3 g
- Peptone - 5 g
- Distilled water - 1000 ml
- Agar - 20 g

The pH was adjusted to 6.4 - 6.8 by using 1 N NaOH solution.

Fresh inoculum was prepared by inoculating the yeasts in MGYP medium (pH 7.0) and incubated in shaker at 150 rpm for 48 h.

The sugar hydrolysates of pretreated *Parthenium* biomass were centrifuged and concentrated to the sugar content of 48.12 g/L. The pH of the hydrolysate was 4.8 and which was adjusted to 5.0 using NaOH. The fermentation of sugar hydrolysates (20 ml) was carried out by adding 10% (v/v) of fresh inoculum and incubated under static condition for 96 h at 30°C. Samples were withdrawn at regular intervals and centrifuged at 10000 rpm for 10 min at 4°C. The cell free supernatant was used to determine the ethanol concentration and residual sugar by DNSA method (Miller, 1959). The pellet was dried at 70°C until constant weight and amount of dry yeast biomass was estimated gravimetrically.
3.10.1 Estimation of ethanol and sugars

Ethanol and sugars from the hydrolysates were estimated using high performance liquid chromatography (HPLC) Waters 515 (Waters Corporation, Milford, MA, USA) equipped with a Waters 2414 refractive index detector (RID). The Aminex HPX-87H column was operated with 5 mM H$_2$SO$_4$ as an mobile phase at a flow rate of 0.5 mL/min and the oven temperature was kept at 40 °C. The ethanol standards were prepared using commercial grade ethanol (Merck, Darmstadt, Germany) and the concentration of ethanol was determined by using a calibration curve obtained from standard ethanol solutions.
4. RESEARCH PAPER I

Comparative efficiency of different pretreatment methods on enzymatic digestibility of *Parthenium* sp.

Abstract

The potential of *Parthenium* sp. as a feedstock for enzymatic saccharification was investigated by using chemical and biological pretreatment methods. Mainly chemical pretreatments (acid and alkali) were compared with biological pretreatment with lignolytic fungi *Marasmiellus palmivorus* PK-27. Structural and chemical changes as well as crystallinity of cellulose were examined through Scanning Electron Microscopy (SEM), Fourier Transform Infra Red (FTIR) and X-Ray Diffraction (XRD) analysis, respectively after pretreatment. Total reducing sugar released during enzymatic saccharification of pretreated substrates was also evaluated. Among the pretreatment methods, alkali (1% NaOH) treated substrate showed high recovery of acid perceptible polymerised lignin (APPL) (7.53 ± 0.5 mg/g) and significantly higher amount of reducing sugar (513.1 ± 41.0 mg/g dry solids) compared to uninoculated *Parthenium* (163.4 ± 21.2) after 48 h of hydrolysis. This is the first report of lignolytic enzyme production from *M. palmivorus*, prevalent in oil palm plantations in Malaysia and its application in biological delignification of *Parthenium* sp. Alkali (1% NaOH) treatment proves to be the suitable method of pretreatment for lignin recovery and enhanced yield of reducing sugar which may be used for bioethanol production from *Parthenium* sp.

Key words: *Marasmiellus palmivorus*; Biological pretreatment; Enzymatic saccharification; Bioethanol; *Parthenium* sp.
4.1 Introduction

The rising prices of crude oil, fluctuations in oil supply and environmental issues have fuelled the development of alternative renewable energy source. Lignocellulosic biomass, the most abundant natural resource available on earth, may serve as a renewable alternative for the production of second generation biofuel (Dashtban et al., 2009). Approx. 200 billion tons of lignocellulosic biomass is generated annually on earth (Chandel et al., 2011). Apart from crop biomass, which are used as fuel and feed, weedy biomass may also serve as sustainable lignocellulosic resource for biofuel production. There are many reports that weeds viz. *Eichhornia crassipes* (Ma et al., 2010), *Panicum virgatum* (Dien et al., 2006; Li et al., 2010), *Lantana camara* and *Prosopis julifera* (Gupta et al., 2011b) can be used as a cheaper feedstock for bioethanol production. The wasteland weed, *Parthenium* sp. (congress grass) which have no particular use and its eradication is a major challenge, has invaded nearly 4.25 million hectares of land in India and caused up to 40% loss of economic yield of agricultural crops (Swati et al., 2013). However, very little work has been reported for bioconversion of this biomass into ethanol.

Lignin component of biomass is recalcitrant therefore pretreatment is an essential step for removal of lignin so that cellulose may be saccharified by cellulase enzyme. During enzymatic hydrolysis, the availability of cellulose is hindered by the presence of lignin which is removed or modified by pretreatment methods. There are several methods of pretreatments, among which chemical pretreatment is the most effective and practiced for industrial applications. Alkali pretreatment can be performed at room temperature (Alvira et al., 2010), it causes minimal sugar loss and is reported to be more effective on agricultural residues than woody materials (Kumar et al. 2009a). On the other hand, acid pretreatment solubilizes the hemicellulosic fraction of the biomass and thus makes the cellulose more accessible to enzymes. This method appears as a favourable method for industrial applications and it can be performed at high and low temperature. Saccharification of dilute sulphuric acid pretreated lignocellulosic biomass results in high sugar yield (Alvira et al., 2010). Recently, biological delignification has been practiced using lignolytic microorganisms, which selectively degrade or modify the lignin to gain access to cellulose and hemicellulose (Salvachua et al., 2011). Biopretreatment is advocated by researchers since it provides the additional advantages of being a cheaper, more environmentally friendly process with less consumption of energy. Several white rot
fungi have been employed for biopretreatment of lignocellulosic biomass (Gupta et al., 2011b; Saritha et al., 2013; Tiwari et al., 2013).

In this study, a promising and novel lignolytic fungus *Marasmiellus palmivorus* PK-27 was isolated from decaying biomass. This is the first report on lignolytic activities from this fungus to the best of our knowledge and investigated for biological delignification of *Parthenium* sp. Chemical and biological pretreatment methods were also compared for their efficiency in delignification of *Parthenium* sp. and release of total reducing sugars from pretreated biomass.

4.2 Materials and methods

4.2.1 Sample collection and Isolation of lignocellulolytic fungi

Various plant samples including leaf litter, barks and rotten fruits were collected from hilly forests including Pachmarhi hills (22.47°N 78.43°E) in Bhopal, India, Almora (29.62°N 79.67°E) in Uttarakhand, India and decaying biomass in river basin of Cauvery (11.37°N 78.20°E) in Tamil Nadu, India. Isolation of lignocellulolytic fungi was done by inoculating the samples on Reese’s mineral medium (RMM) KH$_2$PO$_4$ 2 g; (NH$_4$)$_2$SO$_4$ 1.4 g; KNO$_3$ 1.4 g; MgSO$_4$.7H$_2$O 0.3 g; CaCl$_2$.2H$_2$O 0.3 g; FeSO$_4$.7H$_2$O 5 mg; MnSO$_4$.2H$_2$O 1.6 mg; ZnSO$_4$.7H$_2$O 1.4 mg; CoCl$_2$.6H$_2$O 2 mg; Distilled H$_2$O 1000 ml; pH 4.8 with alkali lignin (Sigma) / Carboxymethyl cellulose (CMC) (1%) as a sole source of carbon. The cultures were further purified and subcultured on Potato Dextrose Agar (PDA) and stored at 4°C for screening of lignolytic and cellulolytic enzymes.

4.2.2 Screening of fungal isolates for hydrolytic enzymes

Isolated fungal strains were screened qualitatively and quantitatively for their ability to produce lignocellulolytic enzymes.

4.2.3 Qualitative determination of lignocellulolytic enzymes

The screening of the fungal isolates to produce cellulolytic enzymes was performed in petri dishes on RMM with 1% CMC by incubation at 30°C for 3 days. To visualize the hydrolysis zone, the plates were flooded with Congo red (1 mg/ml) solution for 15 mins. The Congo red solution was then poured off and the plates were destained by 1 M NaCl solution for 15 mins (Teather and Wood, 1982). For the lignolytic enzymes, the fungal isolates were inoculated in the Basal agar medium (lignin-guaiacol): KH$_2$PO$_4$ 0.5 g; MgSO$_4$.7H$_2$O 0.2 g; NH$_4$NO$_3$ 0.1 g; KCl 0.1 g; FeSO$_4$.7H$_2$O 0.02 g; Ca(NO$_3$)$_2$.4H$_2$O 0.05 g; Malt Extract 2 g; Agar 15 g; Distilled
H₂O 1000 ml. Basal medium was autoclaved and cooled approx. to 55°C when the following were added aseptically before plating the medium: 5 ml of 1M KOH; 0.4 ml of guaiacol; 1g of alkali lignin (Sigma) which was partially dissolved and suspended in 10 ml of Dioxane ( Thorn et al., 1996). The plates were incubated at 30°C for 3 days before observing the chromogenic changes.

4.2.4 Quantitative determination of lignocellulolytic enzymes

All fungal isolates were grown under submerged fermentation in RMM with 1% carrot grass (Parthenium sp.) as a lignocellulosic substrate by inoculating an agar plug (6 mm diameter) of previously grown fungal isolates for the production of lignocellulolytic enzymes. Inoculated flasks were incubated statically at 30°C for 7 days. The supernatant was filtered through filter paper and the filtrate was used for the assay of various extracellular lignocellulolytic enzymes.

4.2.4.1 Cellulolytic Enzymes

Endoglucanase (CMCase; E.C. 3.2.1.4), Filter paper lyase (FPase), xylanase (E.C. 3.2.1.8) and β-D-glucosidase (cellobiase; E.C. 3.2.1.21) were assayed in culture filtrate using substrates CMC, Filter paper, xylan and p-nitrophenyl- β-D-glucopyranoside respectively as described by Ghose (1987) and Wood and Bhat (1988). The enzymatic reaction mixture (1ml) contained 500µl of filtrate and 500 µl of respective substrates along with control. The reaction mixtures were incubated at 50°C for 30 min or 1 h for FPase activity. After the incubation, 3 ml of dinitro salicylic acid (DNSA) reagent was added to stop the reaction and tubes were kept in boiling water bath for 10 mins and cooled. The amount of glucose released was measured spectrophotometrically at 575 nm (Miller, 1959). β-D-glucosidase activity was estimated by adding glycine buffer (pH 10.8) after incubation to stop the reaction and amount of p-nitrophenol was measured at 430 nm. The activity was represented as IU/ml⁻¹. One IU was defined as the amount of enzyme capable of liberating 1 µM of reducing sugar per min under assay conditions.

4.2.4.2 Lignolytic Enzymes

Laccase (E.C. 1.10.3.2) activity was analyzed by using 5mM ABTS (2, 2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) in 50mM sodium citrate buffer (pH 4.8) with appropriate dilution of culture filtrate (Childs and Bardsley, 1975). Lignin peroxidase (LiP; E.C: 1.11.1.14) was assayed in enzymatic reaction mixture containing 50 mM tartaric acid (pH 3.0), 2mM veratryl alcohol and 10 mM H₂O₂ (ε₃₄₀=
9300 M⁻¹cm⁻¹) (Tien and Kirk, 1984). The enzyme activity was expressed in International Unit (IU) as the amount of enzyme oxidizing 1µM of substrate per minute under assay conditions.

**4.2.5 Morphological and Molecular identification of fungal isolates**

On the basis of qualitative and quantitative screening, four most promising isolates were selected and its total DNA was extracted by using ZR Fungal/Bacterial DNA Kit (ZymoResearch Corporation, CA, USA). For the sequence analysis ITS1-5.8-ITS2 region of the fungus was amplified by using universal fungal primers homologous to conserved sequences with the small subunit (SSU) rDNA gene, pITS-1 (5’-TCCGTAGGTGAACCTGCGG-3’) and pITS-4 (5’-TCCTCCGCTTATTGATATGC- 3’) (White et al., 1990). The PCR program was as follows: a) initial incubation at 94°C for 5 min b) followed by 35 cycles (94°C for 50 sec, 55°C for 1 min and 72°C for 90 sec) and c) final extension at 72°C for 10 min. DNA sequencing of amplified fragments of approx. 650 bp was done at Eurofins Genomics India Pvt. Ltd, Bangalore. The comparison of partial sequence of the isolate was done with sequences available by the blast search in the National Centre for Biotechnology Information (NCBI – http://www.ncbi.nlm.nih.gov) database to identify the nearest taxa.

**4.2.6 Biological pretreatment of *Parthenium* with *Marasmiellus palmivorus* PK-27**

Five grams of air dried and chopped *Parthenium* biomass was suspended in 25 ml of RMM with solid to liquid ratio of 1:5 and sterilized at 121°C for 20 min. The sterilized and unsterilized *Parthenium* were inoculated with 5 plugs (6 mm diameter) of one week old fungal mycelium and incubated at 30°C for 7 days. One gram of fresh sample was taken from each flask and suspended in 10ml of 50 mM sodium citrate buffer (pH 4.8) for estimation of lignolytic activity. Fungal colonization of substrates was estimated in terms of N- acetyl glucosamine content (Aidoo et al., 1981). Colonized substrates were washed with 0.1 N NaOH (1:10 ratio) to remove lignin and mycelium followed by washing with distilled water to neutralize the pH. The volume of extracts was made to 50 ml and absorbance was recorded at 205 nm to analyze the extent of delignification (Sluiter et al., 2008). Biologically pretreated biomass was used for saccharification.
4.2.7 Acid and alkali pretreatment of *Parthenium*

Five grams of air dried and chopped sample was mixed with 1% dilute H$_2$SO$_4$ solution (10% w/v) in a 500 ml flask capped with a screw cap lid and heated for 20 min in an autoclave at 121°C. For alkali treatment, 5 g of air dried sample was mixed with 1% sodium hydroxide and kept at static condition for 1 hour. The treated samples were filtered through muslin cloth and washed with tap/distilled water until neutrality. The filtrates of dilute acid and alkali pretreatment were sub sampled and used for recovery of APPLs (Crawford et al., 1983) from extracts by acidification and extent of delignification by measuring absorbance of extracts at 205 nm.

4.2.8 Compositional analysis

The cellulose content of raw and pretreated samples was determined by the method described by Updegraff (1969) and pentosans, klaslon lignin, moisture and ash contents were determined according to TAPPI (1997) method.

4.2.9 Analysis of structural and chemical changes in *Parthenium*

Raw and pretreated solid residues were dried and structural and chemical changes were characterized by analytical techniques such as SEM, XRD and FTIR.

4.2.9.1 SEM analysis

For SEM, the untreated and fungal pretreated samples were gradually dehydrated with acetone gradually increasing the concentration up to 100%. Finally, samples were coated with gold/palladium (Au/Pd) and imaging was done at different magnifications between 500 X to 5000 X with ZEISS (EVOMA 10) Scanning Electron Microscope at 20 kV/ EHT and 10 Pa.

4.2.9.2 XRD analysis

For XRD, samples of particle size less than 1mm were scanned at a speed of 1°/min in the range of 20 between 10° and 35° by using Cu-Kα radiation at 40 kV and 25 mA (PW 1729 Philips X-ray generator with PW 1710 diffractometer). Biomass crystallinity index (CrI) was determined according to method by Segal et al. (1959) as follows:

\[ \text{CrI} = \left( \frac{I_{002} - I_{\text{amorphous}}}{I_{002}} \right) \times 100 \]

in which, $I_{002}$ is the intensity for the crystalline portion of biomass (i.e., cellulose) at about $2\theta = 22.5$ and $I_{\text{amorphous}}$ is the peak for the amorphous portion (i.e., cellulose, hemicelluloses and lignin) at about $2\theta = 16.2$. 
4.2.9.3 FTIR analysis

FTIR spectra was obtained from the finely ground pretreated and untreated samples by pressing uniformly into the disc and the analysis was done using Attenuated Total Reflectance-Fourier Transmission Infra Red (ATR- FTIR) system (ALPHA–E, Bruker) with a resolution of 1 cm\(^{-1}\) over the wavelength range of 4000 to 600 cm\(^{-1}\).

4.2.10 Enzymatic saccharification of fungal and chemical pretreated Parthenium

One gram of raw and pretreated samples were transferred to 50 ml screw capped bottle to which 10 ml of 50mM Citrate buffer (pH- 4.8) at 10% substrate loading, 100 µl of 2% Sodium azide and 0.5 ml of commercial cellulase preparation Accellerase\(^{\circledR}\)1500 were added. The bottles were kept in shaking water bath at 50°C for 48 hours. Aliquots were taken periodically for the estimation of total reducing sugar by DNSA method (Miller, 1959). The extent of saccharification was expressed as mg reducing sugar/g of dry substrate (mggds\(^{-1}\)).

4.3 Results and Discussion

4.3.1 Screening of fungal isolates for lignocellulolytic enzymes

Twenty eight fungal strains were isolated and screened qualitatively and quantitatively for lignocellulolytic enzyme activities. In qualitative assay, all the twenty eight fungal strains had shown clear hydrolytic zones when stained with Congo red and destained by 1 M NaCl on RMM agar plates with 1% CMC which indicate that they were capable of producing cellulolytic enzymes. In case of lignolytic enzyme, only four isolates had shown bright red zone around the fungal colony on Basal (lignin-guaiacol) agar medium plates. The formation of bright red zone was due to the oxidation of guaiacol into quinone by the action of laccase or peroxidase produced by the fungal strains (Supplementary Fig.1).

In quantitative assay, lignocellulolytic enzyme production was analysed after 7 days of incubation in RMM with 1% Parthenium as a substrate under submerged fermentation at 30°C. All the thirty isolates produced cellulolytic activity in a variable range, CMCase (0.03–2.92 IUml\(^{-1}\)), FPase (0.004–0.22 IUml\(^{-1}\)), β-glucosidase (0.02–2.36 IUml\(^{-1}\)) and xylanase (0.10 – 14.75 IUml\(^{-1}\)). Among all the fungal isolates, isolate PK-18 showed maximum activity (2.92 ± 0.02IUml\(^{-1}\)) of CMCase and FPase (0.22 ± 0.01 IUml\(^{-1}\)). Isolate PK-16 showed high β-glucosidase activity (2.36 ± 0.05IUml\(^{-1}\))
and isolate PK-23 produced high xylanase activity (14.75 ± 0.33 IU/ml). The results of four promising isolates are presented in Table 1.

The main aim of this experiment was to select a potent lignolytic fungus that disrupts the plant biomass cell wall matrix in order to expose structural carbohydrates with the minimal loss of cellulose. Therefore, on the basis of qualitative and quantitative screening of all fungal isolates, PK-27 was identified as the most promising isolate having high lignolytic activity and minimal cellulolytic activity. Based on sequencing of ITS region of selected fungal isolates, PK-16, PK-18, PK-27 and PK-23 were identified as Aspergillus niger, A. flavus, Marasmiellus palmivorus and Trichoderma longibrachiatum and their partial sequence was submitted to NCBI GenBank under the accession nos. KC771221, KC771222, KC771224 and KC771225, respectively. M. palmivorus produced high level of lignolytic enzymes with highest laccase activity, under submerged (69.1 ± 5.5 IU/ml) and solid state fermentation (452.89 ± 15.2 IU/ml). These features favoured the selection of M. palmivorus PK-27 for biological pretreatment of weedy biomass. This is the first report regarding the lignocellulolytic enzyme activity from M. palmivorus which is prevalent in oil palm plantations in Malaysia (Pong et al., 2012).

4.3.2 Biological and chemical pretreatment of Parthenium

The pretreatment of biomass leads to solubilization and degradation of polymeric cell wall linked lignin. When these lignin molecules present in the extracts were recovered by acidification, higher amount of APPL was recovered from alkali treated samples (7.53 mg/g) and it was approx. 2 times higher than uninoculated substrates. The high absorbance at 205 nm (0.970 ± 0.17) in alkali treated samples also indicates heavy removal of lignin in alkali treated substrate as compared to microbial treated and uninoculated samples (Table 2). The extent of fungal colonization was quantified in terms of N-acetyl glucosamine per gram of substrate and was compared with control. The biological pretreatment of Parthenium under solid state fermentation with M. palmivorus PK-27 for 7 days resulted in more amounts (216.3 ± 11.6 mg/g) of N-acetyl glucosamine compared to uninoculated Parthenium (111.4 ± 3.0 mg/g) indicating the massive colonization of inoculated fungus over the substrate.

4.3.3 Compositional analysis of Parthenium

The compositional analyses of raw and pretreated samples were described in Supplementary Table 1. The presence of 61.2% and 60.2% of total carbohydrate in
Table 1. Lignocellulolytic activity of promising isolates under submerged fermentation with *Parthenium* sp. (1% w/v) as substrate under static condition for 7 days at 30°C.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Cellulolytic activity (IU/ml)</th>
<th>Xylanase (IU/ml)</th>
<th>Lignolytic activity (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CMCase (Endo β-D glucanase)</td>
<td>FPase (Exo β-D glucanase)</td>
<td>Cellobiase (β-D glucosidase)</td>
</tr>
<tr>
<td>PK-16</td>
<td>1.13 ± 0.24</td>
<td>0.13 ± 0.00</td>
<td>2.36 ± 0.05</td>
</tr>
<tr>
<td>PK-18</td>
<td>2.92 ± 0.02</td>
<td>0.22 ± 0.01</td>
<td>0.82 ± 0.10</td>
</tr>
<tr>
<td>PK-23</td>
<td>0.20 ± 0.02</td>
<td>0.08 ± 0.07</td>
<td>0.40 ± 0.01</td>
</tr>
<tr>
<td>PK-27</td>
<td>0.03 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.08 ± 0.00</td>
</tr>
</tbody>
</table>

Data represents mean ± SD, n=3

*ND- Not Detected
Table 2. The extent of delignification and lignin recovery as acid perceptible polymerised lignin (APPL) by acidification with conc. H$_2$SO$_4$ after the pretreatment of *Parthenium* sp.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Recovery of lignin after pretreatment</th>
<th>Extent of delignification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>APPL (mg/g)</td>
<td>Absorbance of alkali extracts (at 205 nm)</td>
</tr>
<tr>
<td><em>M. palmivorus</em> PK-27</td>
<td>6.13 ± 0.2</td>
<td>0.647 ± 0.02</td>
</tr>
<tr>
<td>Acid (1% H$_2$SO$_4$)</td>
<td>1.00 ± 0.0</td>
<td>0.894 ± 0.05</td>
</tr>
<tr>
<td>Alkali (1% NaOH)</td>
<td>7.53 ± 0.5</td>
<td>0.970 ± 0.17</td>
</tr>
<tr>
<td>Uninoculated</td>
<td>3.67 ± 0.5</td>
<td>0.384 ± 0.06</td>
</tr>
</tbody>
</table>

Data represents mean ± SD, $n=3$
acid and alkali pretreated sample shows the potential of *Parthenium* as a feedstock for fermentable sugar production.

### 4.3.4 Analysis of structural and chemical changes in *Parthenium* sp. due to chemical and biological pretreatment

Dilute acid and alkali pretreatment is mostly exploited for agricultural biomass (Wyman et al., 2005). SEM images of pretreated and untreated samples are shown in Fig. 1. The images of pretreated sample reveal the formation of some holes on the biomass surface and disruption of the biomass matrices which renders cellulose more accessible to the cellulolytic enzymes during saccharification.

Among several features, crystallinity is believed to be a significant factor which affects enzymatic saccharification of glucan (Mansfield et al., 1999; Zhang and Lynd, 2004). The crystallinity index of pretreated and untreated samples was calculated from the XRD data and the results are summarized in Fig. 2. The CrI for untreated sample was 32.5%, which has shown an increase after dilute acid (44.5%) and alkali pretreatment (39.7%). However, the biologically pretreated sample showed decrease in crystallinity of 29%. This increased CrI after dilute acid and alkali pretreatment suggest that under acidic condition there is more break down of amorphous cellulose and also acid treatment is unable to break apart the inter and intra chain hydrogen bonds in cellulose fibrils (Li et al., 2010; Kumar et al., 2009b; He et al., 2008). Similarly, the increased crystallinity index (CrI) of alkali treated sample could be due to increased hydrolyzation and occurrence of peeling reaction in the amorphous region than the crystalline region. Similar results were reported by earlier workers (He et al., 2008; Wu et al., 2011). The decrease in CrI of fungal (*M. palmivorus*) treated sample may attributed to the breakdown of inter/intra hydrogen bonding in the crystalline cellulose resulting in modified crystal structure as reported by Zhang et al. (2012).

FTIR spectra of the untreated and pretreated samples are shown in Figs. 3 and 4. The spectrum for crude lignin appears at two peaks (836 and 1166 cm\(^{-1}\)) which are typical of SGH lignin (Syringyl-Guaiacyl- \(p\)-hydroxyphenyl). Syringyl (S) and Guaiacyl (G) units are detected by vibrations of aromatic skeleton at 1609 cm\(^{-1}\), 1126 cm\(^{-1}\) and 1330 cm\(^{-1}\) (S), 1513 cm\(^{-1}\), 1034 cm\(^{-1}\) and 1265 cm\(^{-1}\) (G) (Zeng et al., 2011). The reduction in the transmittance of above mentioned peaks reveal that lignin was
partially removed in treated biomass compared to untreated substrate. The peak position at 2900 cm\(^{-1}\) and 2860 cm\(^{-1}\) is attributed to C–H stretching (methyl and methylene groups) corresponding to the aliphatic moieties in lignin and polysaccharide. Peak at 2360 cm\(^{-1}\) indicates C=O bonds in ketone groups. The reduction of these peaks in pretreated samples confirms the rupture of the functional groups in lignin/cellulose compared to untreated sample which showed no reduction. The peak at 1726 cm\(^{-1}\) is attributed to the acetyl and uronic ester groups of the hemicelluloses or ester linkage of carboxylic group of the ferulic and p-coumaric acids of lignin and/or hemicelluloses, the absence of this peak in alkali treated sample indicates the cleavage of ester bond from hemicelluloses (Sun et al., 2005). The peak at 1165 cm\(^{-1}\) is attributed to ester bond stretching which disappeared after the alkali pretreatment (Fig. 3), this is consistent with the report of He et al. (2008) suggesting that it could be due to saponification reaction occurred during pretreatment. Overall, the alkali treated substrates showed high reduction of the above mentioned peaks indicating heavy removal/modification of the lignin molecules thus increasing the cellulose digestibility during enzymatic hydrolysis.

### 4.3.5 Enzymatic saccharification of fungal and chemical pretreated *Parthenium*

The total reducing sugar yield from enzymatic saccharification of pretreated and untreated biomass are shown in Fig. 5. The composition of the reducing sugars obtained from the different pretreatments was also determined by HPLC (Supplementary Table 2). The maximum sugar yield was achieved (513.1 ± 41.0 mggd\(^{-1}\)) from alkali pretreated samples. In previous reports also, pretreatment of wheat straw with 2% NaOH released a maximum of 567 mggd\(^{-1}\) of reducing sugar (McIntosh and Vancov, 2011). Gupta et al. (2011a) used 5% NaOH for chemical pretreatment in *Lantana camara* resulting in 508 mggd\(^{-1}\) of reducing sugar. This could be due to swelling, increase of internal surface of cellulose and decrease in degree of polymerization and crystallinity which induces lignin disruption during alkali treatment (Taherzadeh and Karimi, 2008).

In acid pretreated samples the sugar yield was 476.4 ± 30.2 mggd\(^{-1}\). The sugar yield of 565 mggd\(^{-1}\) with 0.75% (v/v) of H\(_2\)SO\(_4\) at 121°C for 1 hour in wheat straw (Saha et al., 2005) and 440 mggd\(^{-1}\) with 1% (v/v) of H\(_2\)SO\(_4\) at 160°C in paddy straw (Hsu et al., 2010) has been reported earlier. Dilute acid hydrolysis at 121°C releases a moderate level of reducing sugars and less inhibitors which favour
fermentation but use of concentrated acid is not desirable because of the formation of fermentation inhibitors (Pasha et al., 2007). Acid and alkali pretreatments have considerable effect on lignin structure and increased accessibility for enzymes. Acid treatment also causes solubilisation of hemicelluloses as well as generation of toxic compounds (Alvira et al., 2010). When compared with acid/alkali pretreatments, biologically pretreated samples resulted in comparably less amount of sugar yield (410.3 ± 45.1mggds⁻¹). It could be due to utilization of cellulose from substrates by fungal biomass during the period of biological pretreatment, which in turns resulted in decreased amount of holocellulose available for enzymatic hydrolysis. However, it has many advantages which include selective degradation of lignin molecules, absence of toxic compounds and less energy consumption.

Several studies conducted with different substrates by using acid/alkali/biological treatments are summarized in Table 3. There are reports on biological delignification of *Parthenium* using *Trametes hirsuta* ITCC 136 and *Myrothecium roridum* LG7 (Tiwari et al., 2013; Rana et al., 2013) but chemical treatment have not been carried out in these studies. The present study proves the superiority of mild alkali treatment as best pretreatment method in case of *Parthenium* sp. for biofuel production.

### 4.4 Conclusion

The present study aimed to provide a comparative analysis of structural, chemical changes and fermentable sugar production from *Parthenium* sp. by using two commonly used chemical pretreatments (acid/alkali) and biological pretreatment with selected lignolytic fungus. An inverse correlation was observed between lignin and enzymatic digestibility. Compared with other two pretreatment methods, sodium hydroxide pretreatment of *Parthenium* sp. substantially increased the lignin recovery as APPL and enhanced the cellulose digestibility and enzymatic accessibility. Moreover, maximum content of fermentable sugars was released from alkali pretreated sample after enzymatic saccharification which makes it more suitable for subsequent fermentation process for ethanol production. Further studies needs to be carried out for optimization of conditions for alkali pretreatment e.g. alkali concentrations, temperature and time etc. to improve enzymatic digestibility of the biomass.
Table 3. Comparison of reducing sugar released from chemical and biological pretreatments with different lignocellulosic substrates

<table>
<thead>
<tr>
<th>Pretreatment substrates</th>
<th>Treatment conditions</th>
<th>Reducing sugar (mggd$^{-1}$)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat straw</td>
<td>(0.75% H$_2$SO$_4$ v/v), 121°C for 1h</td>
<td>565</td>
<td>(Saha et al. 2005)</td>
</tr>
<tr>
<td>Paddy straw</td>
<td>(1% H$_2$SO$_4$ v/v), 160°C for 5 min</td>
<td>440</td>
<td>(Hsu et al. 2010)</td>
</tr>
<tr>
<td>Lantana camara</td>
<td>(3% H$_2$SO$_4$ v/v), 120°C for 45 min</td>
<td>187</td>
<td>(Kuhad et al. 2010)</td>
</tr>
<tr>
<td>Parthenium sp.</td>
<td>(1% H$_2$SO$_4$ v/v), 121°C for 20 min</td>
<td>476</td>
<td>Present study</td>
</tr>
<tr>
<td>Alkali</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat straw</td>
<td>(2% NaOH), 121°C for 1.5h</td>
<td>567</td>
<td>(McIntosh and Vancov 2011)</td>
</tr>
<tr>
<td>Paddy straw</td>
<td>(2% NaOH), 85°C for 1h</td>
<td>685</td>
<td>(Jeya et al. 2009)</td>
</tr>
<tr>
<td>Lantana camara</td>
<td>5% NaOH for 2h and 121°C for 30 min</td>
<td>508</td>
<td>(Gupta et al. 2011)</td>
</tr>
<tr>
<td>Parthenium sp.</td>
<td>(1% NaOH), room temp. for 1h</td>
<td>513</td>
<td>Present study</td>
</tr>
<tr>
<td>Biological</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat straw</td>
<td>(Fungal isolate RCK-1), 10 days</td>
<td>408*</td>
<td>(Kuhar et al. 2008)</td>
</tr>
<tr>
<td>Paddy straw</td>
<td>(Streptomyces griseorubens SSR38), 10 days</td>
<td>380</td>
<td>(Saritha et al. 2013)</td>
</tr>
<tr>
<td>Lantana camara</td>
<td>(Pycnoporus cinnabarinus), 15 days</td>
<td>402</td>
<td>(Gupta et al. 2011)</td>
</tr>
<tr>
<td>Parthenium sp.</td>
<td>(Myrothecium roridum LG7), 7 days</td>
<td>455</td>
<td>(Tiwari et al. 2013)</td>
</tr>
<tr>
<td>Parthenium sp.</td>
<td>(Marasmiellus palmivorus PK-27), 7 days</td>
<td>410</td>
<td>Present study</td>
</tr>
</tbody>
</table>

*value is converted into mg/g substrate from 40.8 g/L as reported in literature
Acknowledgements

Authors are thankful to Post Graduate School and Director, Indian Agricultural Research Institute (New Delhi) for providing fellowship to carry out Ph.D. programme of first author and National Fund for Basic, Strategic and Frontier Application Research in Agriculture. We also thank Dr. V.V. Ramamurthy, Principal Scientist, Division of Entomology, Dr. Madhuban Gopal, National Fellow, Principal Scientist, Division of Agricultural Chemicals and Dr. S.C. Dutta, Principal Scientist, Division of Soil Science, IARI for providing SEM, FTIR and XRD facility, respectively.
Figure legends

**Fig.1.** SEM images at various magnifications for pretreated and raw *Parthenium* sp. [A - *M. palmivoros* treated (535X), B - Acid (1% H$_2$SO$_4$) treated (598X), C - Alkali (1% NaOH) treated (932X), D– Raw *Parthenium* (3.68kX)].

**Fig.2.** XRD pattern of chemical (1% NaOH and 1% H$_2$SO$_4$), microbial (*M. palmivoros*) pretreated and untreated *Parthenium* to deduce the changes in cellulose crystallinity

**Fig.3.** FTIR spectra of chemical (1% NaOH and 1% H$_2$SO$_4$) pretreated and untreated *Parthenium* depicts the changes in the composition of biomass

**Fig.4.** FTIR spectra of microbial (*M. palmivoros*) pretreated and untreated *Parthenium* indicating changes in the composition of biomass

**Fig.5.** Total reducing sugars during the enzymatic saccharification of pretreated and untreated *Parthenium* at 50°C for 48h by using commercial enzyme Accellerase® 1500

**Supplementary Fig. 1.** Plate shows the formation of bright red zone around the fungal disc producing lignolytic enzymes along with negative control

**Supplementary Table 1.** Chemical composition of raw and pretreated *Parthenium* sp.

**Supplementary Table 2.** Composition of reducing sugars in the enzymatic hydrolysates of different pretreatments was determined by HPLC
Fig. 1. SEM images at various magnifications for pretreated and raw *Parthenium* sp. [A - *M. palmivorus* treated (535X), B - Acid (1% H$_2$SO$_4$) treated (598X), C - Alkali (1% NaOH) treated (932X), D - Raw *Parthenium* (3.68kX)].
Fig. 2 XRD pattern of chemical (1% NaOH and 1% H$_2$SO$_4$), microbial (M. palmivorus) pretreated and untreated Parthenium to deduce the changes in cellulose crystallinity
**Fig. 3** FTIR spectra of chemical (1% NaOH and 1% H$_2$SO$_4$) pretreated and untreated *Parthenium* depicts the changes in the composition of biomass.
Fig. 4 FTIR spectra of microbial (*M. palmivorus*) pretreated and untreated *Parthenium* indicating changes in the composition of biomass.
Fig. 5 Total reducing sugars during the enzymatic saccharification of pretreated and untreated Parthenium at 50°C for 48 h by using commercial enzyme Accellerase®1500
**Supplementary Table 1.** Chemical composition of *Parthenium* sp. before and after pretreatment

<table>
<thead>
<tr>
<th>Composition (%)</th>
<th>Raw sample</th>
<th>Acid treated</th>
<th>Alkali treated</th>
<th><em>M. palmivorus</em> treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>30.8 ± 0.6</td>
<td>48.1 ± 1.4</td>
<td>40.2 ± 0.4</td>
<td>33.6 ± 1.0</td>
</tr>
<tr>
<td>Pentosans</td>
<td>16.4 ± 0.2</td>
<td>13.1 ± 0.3</td>
<td>20.0 ± 0.3</td>
<td>14.0 ± 0.1</td>
</tr>
<tr>
<td>Klason lignin</td>
<td>18.1 ± 1.0</td>
<td>21.7 ± 0.6</td>
<td>15.1 ± 0.5</td>
<td>15.5 ± 0.4</td>
</tr>
<tr>
<td>Ash</td>
<td>8.7 ± 0.3</td>
<td>1.8 ± 0.1</td>
<td>6.1 ± 0.1</td>
<td>10.0 ± 0.2</td>
</tr>
</tbody>
</table>

Data represents the mean ± SD
**Supplementary Table 2.** Composition of reducing sugars obtained from enzymatic hydrolysates of different pretreatments was determined by HPLC

<table>
<thead>
<tr>
<th>Sugars (mg/ml)</th>
<th>Alkali treated</th>
<th>Acid treated</th>
<th><em>M. palmivorus</em> treated</th>
<th>uninoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>5.20 ± 0.27</td>
<td>5.14 ± 0.08</td>
<td>3.86 ± 0.3</td>
<td>2.82 ± 0.11</td>
</tr>
<tr>
<td>Xylose</td>
<td>2.14 ± 0.16</td>
<td>1.43 ± 0.04</td>
<td>1.41 ± 0.18</td>
<td>1.14 ± 0.02</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0.05 ± 0.0</td>
<td>0.00</td>
<td>0.01 ± 0.0</td>
<td>0.001 ± 0.0</td>
</tr>
</tbody>
</table>

Data represents the mean ± SD
Supplementary Fig. 1 Plate shows the formation of bright red zone around the fungal disc producing lignolytic enzymes along with negative control
5. RESEARCH PAPER II

Optimization of enzymatic saccharification of alkali pretreated *Parthenium* sp.
using response surface methodology

Abstract

*Parthenium* sp. is a noxious weed which threatens the environment and biodiversity due to its rapid invasion. This lignocellulosic weed was investigated for bioethanol production by subjecting it to mild alkali pretreatment followed by enzymatic saccharification which resulted in significant amount of sugar yield (76.6%). Optimization of enzymatic hydrolysis variables such as temperature, pH, enzyme and substrate loading was carried out using central composite design (CCD) in response surface methodology (RSM) to achieve the maximum saccharification yield. Data obtained from RSM was validated using ANOVA. After the optimization process, a model was proposed with predicted value of 80.08% saccharification yield under optimum conditions which was confirmed by the experimental value of 85.80%. This illustrated a good agreement between predicted and experimental response (saccharification yield). The saccharification yield was enhanced by enzyme loading and reduced by temperature and substrate loading. This study reveals that under optimized condition, sugar yield was significantly increased which was higher than earlier reports and promises the use of *Parthenium* sp. biomass as a feedstock for bioethanol production.

Key words: Optimization; Enzymatic saccharification; Response surface methodology; Bioethanol; *Parthenium* sp.
5.1 Introduction

In the last few decades, the demand for alternative fuel sources is accelerated due to the excessive consumption of fossil fuels (Zaldivar et al., 2001). Currently, ethanol production process uses crops such as sugar cane and corn but they have social issues associated with the exploitation of potential food or feed resources (Ferreira et al., 2009). Therefore, the utilization of non-food biomass i.e. lignocellulosic biomass is creating interest worldwide. The lignocellulosic biomass has the advantage of huge availability, economical, reduced emissions of greenhouse gases and does not have the socio-economical concerns regarding the use of food resources. These factors make them as one of the most promising technological approaches available for supplementing the current source of transportation fuel. Effective conversion of recalcitrant lignocellulosic biomass to ethanol includes five subsequential steps: (1) biomass pretreatment (2) cellulose hydrolysis (saccharification) (3) fermentation of hexoses (4) separation and (5) effluent treatment (Cardona and Sanchez, 2007).

*Parthenium* sp., belonging to the family Asteraceae, is native to the American tropics and commonly known as congress grass/carrot weed or gazar ghas. In India, this weed poses serious threat to biodiversity by replacing the indigenous species. At present this invasive weed has infested about 35 million ha of land in India since its first introduction in 1955 (Sushilkumar, 2009). It is able to grow on wide range of soil types ranging from sandy to heavy clay soil, but better growth is observed in moist type of soil. It occurs in areas with summer rainfall greater than 500 mm per annum (Shubhaneel, 2013). The excessive growth rate and wider adaptability of this weed shows it as a potential renewable source of lignocellulosic biomass available for ethanol production.

Pretreatment process is essential for removal of lignin and hemicelluloses, to reduce cellulose crystallinity and increase the porosity of biomass (Alvira et al., 2010). Saccharification is the critical step for sugar production. Enzymatic saccharification of cellulosic biomass has been considered as an environmentally friendly process that replaces chemical treatment of acid (sulphuric acid) saccharification (Jeya et al., 2009). The main bottleneck for the commercialization of bioethanol is due to high costs of the two processes, pretreatment and enzymatic hydrolysis (Ruiz et al., 2008).
Saccharification is an important step for maximum sugar yield, with enzyme and substrate loading, pH and temperature constituting important parameters for optimization of saccharification process. Optimization of multi factorial system by conventional techniques is generally done with one-factor at a time. However, this type of method is time consuming and does not reveal the interactive effects between the variables (Jeya et al., 2009). Response surface methodology (RSM) is a statistical technique for the modelling and optimization of multiple variables, which determines the optimum process conditions through combining experimental designs with interpolation by first- or second-order polynomial equations in a sequential testing procedure (Ferreira et al., 2009).

In the present study, *Parthenium* biomass was used as a substrate for ethanol production by subjecting it into mild alkali pretreatment and enzymatic saccharification. An attempt was also made to apply RSM to identify the optimum process conditions for sugar production from *Parthenium* biomass by developing experimental design (CCD) and analyzing the influence and interactions of variables during saccharification.

5.2 Materials and methods

5.2.1 Pretreatment of Parthenium

The *Parthenium* biomass was collected from Indian Agricultural Research Institute (IARI) farm in the month of May, air dried and chopped into small size (2-5 cm) and stored in an airtight polythene bag at room temperature until further use. The pretreatment was carried out by suspending the substrate with 50 ml of 1% NaOH (1:10 solid to liquid ratio) and kept at static condition for 1 h. The pretreated substrate was filtered using muslin cloth and the remaining solid residue was washed with distilled water until the neutral pH. The pretreated substrate was dried at 60°C and its composition was determined.

5.2.2 Compositional analysis

The cellulose content of raw and pretreated biomass was determined by the method described by Updegraff (1969) and pentosans, klason lignin, moisture and ash contents were determined according to TAPPI (1997) method.
5.2.3 Saccharification of pretreated substrate

Saccharification of pretreated substrate was performed in 50 ml screw capped bottles, on a rotary shaker. A set up comprising 10 ml reaction mixture in 50 mM sodium citrate buffer was prepared according to the experimental design, with supplementation of 100 µl of 2% sodium azide to prevent microbial contamination. The enzyme complex used for hydrolysis was Accellerase®1500 (Endoglucanase: 2200-2800 CMC U/g, β-glucosidase: 525-575 pNPG U/g). Samples were taken from the reaction mixture at different time intervals and centrifuged at 10000 rpm for 5 min. The supernatant was used for analysis of reducing sugar by DNSA method (Miller, 1959) and the saccharification efficiency was calculated as saccharification (%):

\[
\text{Saccharification yield (\%)} = \frac{\text{Reducing sugars released (mg) } \times 0.9}{\text{Carbohydrate content in pretreated biomass (mg)}} \times 100
\]

5.2.4 Design of experiment

The sugar yield of enzymatic hydrolysis in terms of saccharification efficiency was taken as the response influenced by many potential variables. In this study, a central composite rotatable design (CCRD) was employed to determine the effects of independent variables on response and to optimize the enzymatic hydrolysis. Thirty runs of experiment were formed by Design Expert® 8.0.7.1 version (Stat-Ease, Inc., Minneapolis, USA) with six replications at the central point and eight replications at axial and sixteen replications at factorial points. The variables include temperature (A), pH (B), enzyme loading (C) and substrate loading (D). The coded and decoded values are listed in Table 1.

Using Design Expert® 8.0.7.1, all the statistical and mathematical analysis of the results was done to evaluate the effects of variables and their interactions. Three dimensional surface plots were drawn to show the effects of independent variables on response and a quadratic polynomial equation was proposed to describe the mathematical relationship between the variables and the response. The significance of the model was evaluated by determination of $R^2$ and adjusted $R^2$ coefficient. Also an experiment was conducted to confirm the predicted optimum response using the selected optimum values of the four variables.
Table 1 Coded and decoded values for each variables of Central Composite Design (CCD)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Coded levels of the experimental variables</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-α</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>45</td>
</tr>
<tr>
<td>pH</td>
<td>4.0</td>
</tr>
<tr>
<td>Enzyme loading (ml)</td>
<td>0.2</td>
</tr>
<tr>
<td>Substrate loading (g)</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Table 2 Compositional analysis of raw and alkali pretreated *Parthenium* sp.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Raw sample (%)</th>
<th>Alkali treated (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>30.8 ± 0.6</td>
<td>40.2 ± 0.4</td>
</tr>
<tr>
<td>Pentosans</td>
<td>16.4 ± 0.2</td>
<td>20.0 ± 0.3</td>
</tr>
<tr>
<td>Klason lignin</td>
<td>18.1 ± 1.0</td>
<td>15.1 ± 0.5</td>
</tr>
<tr>
<td>Ash</td>
<td>8.7 ± 0.3</td>
<td>6.1 ± 0.1</td>
</tr>
<tr>
<td>Moisture</td>
<td>6.0 ± 0.4</td>
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</table>

Data represents mean ± SD
5.3 Results and discussion

5.3.1. Compositional analysis of *Parthenium*

The compositional analysis of pretreated samples revealed that the biomass contain cellulose (40.2 ± 0.4%); pentosans (20.0 ± 0.3%); klason lignin (15.1 ± 0.5%); moisture (6.1 ± 0.1%) and ash (6.5 ± 0.3%). The presence of 60.2 % of total carbohydrate content and the abundant nature shows the potential of *Parthenium* as a feedstock for bioethanol production.

5.3.2. Saccharification of pretreated substrates

Saccharification is a key step in the conversion of cellulose into sugar. Lignocellulosic biomass cannot be saccharified by enzymes without pretreatment as the presence of lignin in plant cell wall act as a barrier for enzymatic activity (Sewalt et al., 1997). The substrate was pretreated with 1% NaOH prior to enzymatic hydrolysis which increased the proportion of cellulose and pentosans by 30.5 and 22%, respectively and reduced the lignin content by 16.6% in the biomass (Table 2).

Initially the enzymatic hydrolysis was carried out by using 0.1 g of pretreated substrate (*Parthenium* sp.) in 10 ml of 50 mM sodium citrate buffer (pH 4.8) with 0.5 ml commercial enzyme complex, Accellerase®1500 and incubated at 50°C. This resulted in maximum sugar release of 513 mg per gram of dry substrate (76.6%) after 48 h of hydrolysis.

5.3.3. Optimization of hydrolysis parameters for increased sugar yield

5.3.3.1. Development of a model for enzymatic saccharification

Response surface methodology (RSM), a collection of statistical and mathematical techniques, is normally used for modelling and analyzing problems in which several variables influencing the response of interest may be tested and the aim is to optimize the response (Ferreira et al., 2009). To improve the sugar yield and rate of saccharification, research was focused on optimizing the hydrolysis process. Saccharification efficiency, the response which influenced by temperature, pH, enzyme loading and substrate loading was evaluated. On the basis of initial results from enzymatic hydrolysis, the conditions for optimizing the hydrolysis are follows: temperature, 45 - 65°C; pH, 4 - 5; enzyme loading, 0.2 - 1.0 ml and substrate loading, 0.1 - 0.5 g which are summarized in Table 3. The buffer used for
Table 3: Experimental design and results of CCD for enzymatic hydrolysis of pretreated Parthenium sp.

<table>
<thead>
<tr>
<th>Run No.</th>
<th>A: Temperature (°C)</th>
<th>B: pH</th>
<th>C: Enzyme loading (ml)</th>
<th>D: Substrate loading (g)</th>
<th>Saccharification Efficiency (%)</th>
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<tr>
<td></td>
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<td>Experimental</td>
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<tr>
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<tr>
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<tr>
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<td>0.3</td>
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<tr>
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<td>0.6</td>
<td>0.3</td>
<td>54.73</td>
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</table>
enzymatic hydrolysis was sodium citrate buffer but the range of pH was modified according to the experimental design.

The coefficient of determination ($R^2$) of the model was 0.96 (Table 4). $R^2$ is a measure of the amount of variation around the mean explained by the model. From the above $R^2$ value, it was shown that only 4% of the variation for response could not be explained by the model. The $R^2$ value (0.96) was in good agreement with the adjusted $R^2$ value (0.93) and well adapted to the response, also the predicted $R^2$ value (0.78) was in reasonable agreement with adjusted $R^2$ value. The coefficient of variation (CV %) of 12.91 and standard deviation (SD) 5.53 were relatively low and acceptable. The coefficient of variation (%) is the measure of residual variation of the data relative to the size of the mean and is inversely related to the experiment reliability (Saini et al., 2013). Adequate precision, measures the signal to noise (S/N) ratio and the values greater than 4 is desirable. The S/N ratio of 18.62 indicated the adequate signal and the model can be used to navigate the design space.

ANOVA was carried out to determine the significance of the model and model terms (Table 4). The model F-value of 26.51 implied that the model was significant and there was only a 0.01% chance that the model F-value could occur due to noise. The $p$-value is normally used as a tool to check the significance of each of the coefficients. Smaller the $p$-value, correlation is more significant with the corresponding coefficient. The “$p$-value” for the model was < 0.0001, which indicated that the model was statistically significant and the $p$-value for model terms $A$, $C$, $D$, $AD$, $A^2$, $B^2$ and $C^2$ were less than 0.05 indicating that they were the significant variables influencing the response (saccharification %) than the others. The absence of interactions between variables ($p > 0.05$) except $AD$ can be assumed to be an additive effect of these variables on the response (Ferreira et al., 2009). $AD$ was also a significant variable with $p$-value of 0.0289 demonstrating that there was interaction existing between temperature and substrate loading.

The overall second-order polynomial equation (Eq.1) describes the relationship between the variables and the sugar yield from enzymatic hydrolysis of pretreated *Parthenium* sp. in terms of coded values:

$$Y = +54.73 - 17.84 \times A - 1.33 \times B +6.94 \times C -4.71 \times D -0.83 \times AB -2.32 \times AC +3.34 \times AD +1.50 \times BC +0.21 \times BD -0.93 \times CD -3.78 \times A^2 -3.24 \times B^2 -0.94 \times C^2 -6.91 \times D^2$$

- Eq. (1)
Table 4 ANOVA for Quadratic response surface model (RSM) from enzymatic saccharification of pretreated *Parthenium* sp.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>Degree of freedom</th>
<th>Mean square</th>
<th>F value</th>
<th>p-value (Prob&gt;F)</th>
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</thead>
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<tr>
<td>Model</td>
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<td>14</td>
<td>810.04</td>
<td>26.51</td>
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</tr>
<tr>
<td>A: Temperature</td>
<td>7640.87</td>
<td>1</td>
<td>7640.87</td>
<td>250.04</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>B: pH</td>
<td>42.69</td>
<td>1</td>
<td>42.69</td>
<td>1.40</td>
<td>0.2556</td>
</tr>
<tr>
<td>C: Enzyme loading</td>
<td>1155.07</td>
<td>1</td>
<td>1155.07</td>
<td>37.83</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>D: Substrate loading</td>
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<td>533.46</td>
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<tr>
<td>AB</td>
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<td>10.91</td>
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<td>0.5591</td>
</tr>
<tr>
<td>AC</td>
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<td>86.26</td>
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<tr>
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<tr>
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<td>391.03</td>
<td>12.80</td>
<td>0.0028</td>
</tr>
<tr>
<td>B²</td>
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<td>287.84</td>
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<tr>
<td>C²</td>
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<td>24.47</td>
<td>0.80</td>
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<td>D²</td>
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<td>Residual</td>
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<td>15</td>
<td>30.56</td>
<td></td>
<td></td>
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<tr>
<td>Lack of fit</td>
<td>458.38</td>
<td>10</td>
<td>45.84</td>
<td></td>
<td></td>
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<tr>
<td>Pure error</td>
<td>0.00</td>
<td>5</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11798.95</td>
<td>29</td>
<td>391.03</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SD                  5.53  \[ R^2 \] 0.96
Mean              42.83  Adjusted \[ R^2 \] 0.93
CV (%)           12.91  Predicted \[ R^2 \] 0.78
where the coded variables were: Y - Saccharification (%); A - temperature (°C); B -pH; C - enzyme loading (ml) and D - substrate loading (g).

5.3.3.2. Influence of variables on saccharification yield

To analyze the interaction of variables and to determine the optimum value of each variable for maximum saccharification yield, three dimensional response surface curves were drawn against two experimental variables while the other variables were maintained constant at its central level (Fig. 1-6). The effect of incubation temperature and pH, when enzyme loading and substrate loading were at their central level, 0.6 ml and 0.3 g, respectively are shown in Fig. 1. The increase in temperature resulted in low saccharification yield. This could partially be explained by the loss of enzyme activity due to thermal inactivation. The decrease in activity of Accellerase®1000 with increase in temperature during saccharification has been reported earlier (Karki et al., 2011), while the pH showed a minimum reduction at low pH level.

Fig. 2 presents the influence of enzyme loading while pH (4.50) and substrate loading are at their central level. Observations indicated an increase in saccharification yield when the enzyme loading was increased; similar results were reported in earlier studies (Chen et al., 2007; Karki et al., 2011). In general, high enzyme loading results in better hydrolysis probably by increasing the rate and yield of enzymatic hydrolysis. Partially, it could be due to increase in the ratio of total substrate to total enzyme in enzyme loading (Huang and Penner, 1991). Further increase in the enzyme loading may have no significance in the saccharification yield (Zhang et al., 2010). This could be due to the decrease in extent of adsorbed enzyme, transformation of cellulose structure into a less digestible form and inhibition of enzyme activity by accumulated hydrolysis products (Lee and Fan, 1982).

Fig. 3 depicts the influence of substrate loading on saccharification yield while the other variables were kept at its central level. The increase in substrate loading resulted in reduction of saccharification yield. This indicates that the higher substrate load led to problem in mixing by reducing the aqueous phase in the mixture and thereby hindered enzymatic hydrolysis (Xu et al., 2007).

Fig. 4-6 shows the interaction of variables enzyme loading, substrate loading and pH and these interactions on enzymatic saccharification. The substrate loading and enzyme loading variables are interdependent and showed a significant interaction.
Fig. 1-6: Response surface plots of central composite design for optimization of the enzymatic hydrolysis of alkali pretreated *Parthenium* sp. Fig shows the interaction between (1) Temperature and pH; (2) Temperature and Enzyme loading; (3) Temperature and Substrate loading; (4) pH and Enzyme loading; (5) pH and Substrate loading; (6) Enzyme loading and Substrate loading.
between these parameters on saccharification yield. The pH did not show any significant effect on response while interacting with enzyme loading and substrate loading. The influence was solely due to the interacting variable i.e. either enzyme or substrate loading. It might be due to the adaptability of cellulase enzyme complex for the pH range from 4 to 5 which was selected in this study.

Among the variables studied for optimization, enzyme loading, substrate loading and temperature have more effects on the saccharification yield of pretreated *Parthenium* sp. Increase in enzyme loading resulted in enhancement but increase in substrate loading and temperature led to decline of saccharification yield. However, the change in pH (4-5) did not show any major influence on the response (saccharification yield).

5.3.3.3. Optimization of saccharification yield (%)

Optimization of saccharification was carried out numerically by using Design Expert software, version 8.0.7.1, to evaluate the optimum values for each variable from the model. In optimization process, the goal for each variable was to select a range which could provide highest saccharification yield. On the basis of experimental design and developed model, the optimal conditions to maximize the saccharification yield were obtained. The maximum response could be obtained at middle of the temperature, pH and substrate loading and at the higher enzyme loading. The predicted maximum saccharification yield was 80.08% during enzymatic hydrolysis under the optimum conditions i.e. temperature - 50°C; pH - 4.53; enzyme loading - 0.80 ml and substrate loading - 0.24 g (Table 5).

To validate the predicted saccharification yield, an experiment was conducted in triplicate with above mentioned optimum conditions of each variable as developed by the model. The experimental result of response (saccharification %) for pretreated *Parthenium* sp. was 85.80% and it was in good agreement with predicated value of 80.08% for saccharification yield and showed that the model was useful for predicting the optimal conditions for variables influencing the saccharification yield (Table 5).

Several studies have been reported saccharification using various pretreatment methods for different lignocellulosic materials. The saccharification yield during enzymatic hydrolysis of different substrates as reported by other workers is summarized in Table 6. The results revealed the superiority of *Parthenium* biomass
Table 5 Optimum values of variables for enzymatic saccharification of pretreated *Parthenium* sp.

<table>
<thead>
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<th>Variables</th>
<th>Goal</th>
<th>Optimum levels</th>
<th>Desirability</th>
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<tr>
<td>Temperature (°C)</td>
<td>In range</td>
<td>50</td>
<td>0.957</td>
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<tr>
<td>pH</td>
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<td>4.53</td>
<td>-do-</td>
</tr>
<tr>
<td>Enzyme loading (ml)</td>
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<td>-do-</td>
</tr>
<tr>
<td>Substrate loading (g)</td>
<td>In range</td>
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<td>-do-</td>
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<table>
<thead>
<tr>
<th>Response</th>
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<th>Predicted value</th>
<th>Observed value</th>
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<tr>
<td>Saccharification (%)</td>
<td>Maximize</td>
<td>80.08</td>
<td>85.80</td>
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Table 6 Saccharification yields of different lignocellulosic substrates after mild alkali (NaOH) pretreatment based on available literature and this study.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Pretreatment conditions</th>
<th>TRS * (mggds⁻¹)</th>
<th>Saccharification %</th>
<th>References</th>
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</thead>
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<tr>
<td>Wheat straw</td>
<td>1% NaOH, 60°C for 1 h</td>
<td>525</td>
<td>-</td>
<td>McIntosh &amp; Vancov (2011)</td>
</tr>
<tr>
<td>Rice straw</td>
<td>2% NaOH, 110°C for 1 h</td>
<td>-</td>
<td>82%</td>
<td>Ioelovich &amp; Morag (2012)</td>
</tr>
<tr>
<td>Coastal Bermuda grass</td>
<td>1% NaOH, 121°C for 30 min</td>
<td>500</td>
<td>86%</td>
<td>Wang et al. (2008)</td>
</tr>
<tr>
<td>Switch grass</td>
<td>1% NaOH, 121°C for 30 min</td>
<td></td>
<td>86.5%</td>
<td>Wang et al. (2012)</td>
</tr>
<tr>
<td><em>Parthenium</em> sp.</td>
<td>1% NaOH, room temp. for 1 h</td>
<td>574</td>
<td>85.8%</td>
<td>This study</td>
</tr>
</tbody>
</table>

*TRS – Total reducing sugars
in yielding highest amount of sugars under optimized conditions. In addition to this, it also affirms probable reduction in cost of saccharification with minimum energy requirement, since pretreatment was carried out at room temperature (40-45°C). It also confirms the validity of RSM as compared to conventional methods of optimization (Jeya et al., 2009). The optimum process parameters along with mild alkali pretreatment at room temperature has the additional advantage of producing a clean substrate which is highly digestible and rich in cellulose and pentosans (Chen et al., 2013). In addition to this, availability of substrate without overhead costs makes the finding of this investigation a promising approach for bioethanol production.

5.4 Conclusion

The potential of Parthenium sp. as a source for bioethanol production was evaluated by estimating the sugar yield during enzymatic saccharification. To optimize the experimental variables of enzymatic hydrolysis for maximization of saccharification yield, CCD was employed under RSM. This experimental design converts the process variable correlations into a mathematical model which predicts the location of response. From the results it can be concluded that saccharification yield was mainly enhanced by enzyme loading in the given range and inversely affected by temperature and substrate loading. The pH had a neutral effect on the response. Under the optimum conditions, the predicted saccharification yield of 80.08%, was in good agreement with the experimental results of 85.80% and validated the model generated by RSM.

Acknowledgements

The authors are thankful to Post Graduate School and Director, Indian Agricultural Research Institute, New Delhi for providing fellowship towards Ph.D. programme and National Fund for Basic, Strategic and Frontier Application Research in Agriculture (ICAR).
**Figure legends**

**Fig.1-6:** Response surface plots of central composite design for optimization of the enzymatic hydrolysis of alkali pretreated *Parthenium* sp. Fig shows the interaction between (1) Temperature and pH; (2) Temperature and Enzyme loading; (3) Temperature and Substrate loading; (4) pH and Enzyme loading; (5) pH and Substrate loading; (6) Enzyme loading and Substrate loading.
6. RESEARCH PAPER III

Bioethanol production from mild alkali pretreated *Parthenium* sp. by mono-, co-culture fermentation with *Saccharomyces cerevisiae* and *Pichia stipitis*

Abstract

*Parthenium* sp. biomass, a cheap and abundant resource, was evaluated as a feedstock for bioethanol production. The pretreatment of substrate with mild alkali (1% NaOH) prior to enzymatic hydrolysis resulted in 16.6% of lignin removal. Enzymatic hydrolysis of pretreated substrate with commercial enzyme complex Accllerase®1500 produced a maximum of 577.5 ± 21.3 mg/gds total reducing sugars with 86.3% saccharification efficiency within 48 h at 50°C. Monoculture fermentation of enzymatic hydrolysate with *Saccharomyces cerevisiae* LN1 and *Pichia stipitis* NCIM3498 resulted in ethanol concentration of 12.89 ± 0.23 g/L, 10.98± 0.28 g/L and ethanol yield of 0.27 g/g, 0.23 g/g after 36 h, 72 h, respectively. The co-culture fermentation with both yeasts enhanced the ethanol concentration (14.28± 0.31 g/L) as well as ethanol yield (0.30 g/g). This study proves the potential of weedy biomass of *Parthenium* sp. for bioethanol production.

Key words: *Parthenium* sp.; Alkali pretreatment; Enzymatic saccharification; Fermentation; Bioethanol.
6.1 Introduction

Lignocellulosic biomass is the most abundant renewable energy resource available for ethanol production (Lin and Tanaka, 2006). The lignocellulosic biomass should be processed first by acid or enzymatic hydrolysis to release fermentable sugars for biofuel production. The enzymatic hydrolysis is advantageous over acid hydrolysis (conc. H$_2$SO$_4$) due to high conversion efficiency, absence of substrate loss and lack of inhibitory compounds. Moreover, enzymatic hydrolysis can be carried out under moderate and non-corrosive conditions e.g. low temperature and neutral pH (Harmsen et al., 2010). The major challenge in enzymatic hydrolysis of cellulose is its low accessibility due to association with lignin. Therefore, an efficient pretreatment method is necessary to improve enzymatic saccharification (Zhao et al., 2008a). The main aim of the pretreatment is to disrupt the lignocellulose structure for the improvement of cellulose and hemicelluloses accessibility for the action of hydrolytic enzymes (Salvachua et al., 2011). Among several chemical pretreatments, alkali pretreatment causes minimal sugar loss and reported to be more effective on several agricultural residues (Kumar et al., 2009b) e.g. rice straw (Jeya et al., 2009), switch grass (Xu et al., 2010), sweet sorghum bagasse (Wu et al., 2011), Saccharum spontaneum (Chaudhary et al., 2012) and corn stover (Li et al., 2012).

Parthenium sp. commonly known as congress/carrot grass or gazar ghas, is native to tropical America and poses serious threat to biodiversity by replacing the indigenous plants. In India, the weed has invaded most of the tropical and subtropical parts and accounts for 35 million ha of land since its first notice in 1955 (Sushilkumar, 2009). The abundance of Parthenium sp. (approximately 40 tonnes/ha) projects a huge availability of the biomass (Saravanane et al., 2012) and has been studied as a substrate for the production of biogas and enzymes (Gunaseelan, 1995; Dwivedi et al., 2009). These features make it as a potential feedstock for bioethanol production. There are reports on using Parthenium sp. as a feedstock for the production of hydrolytic enzymes and fermentable sugars (Tiwari et al., 2013; Rana et al., 2013).

Glucose and xylose are the two major sugars in the lignocellulosic hydrolysates (Chandrakant and Bisaria, 1998) and there is no single organism that can efficiently ferment these sugars to ethanol (Fu and Peiris, 2008). For the overall utilization of the sugar portions of lignocellulose, a co-culture system in which
potential hexose-fermenting yeast is cultivated with pentose-fermenting yeast is useful (Taniguchi et al., 1997). *Saccharomyces cerevisiae*, the widely studied yeast for hexose fermentation, is unable to utilize pentose sugars. Among the pentose fermenting yeasts *Pichia stipitis* and *Candida shehatae* and *Pachysolen tannophilus* are proven to be highly efficient (Abbi et al., 1996). Co-cultivation of both the yeasts results in high ethanol yields from saccharified lignocellulosic hydrolysates as almost all the sugars are converted to ethanol (Yadav et al., 2011). Therefore, for an efficient conversion of all sugars to ethanol, a co-fermentation of both hexoses and pentoses is a pre-requisite for high ethanol production.

In this study, *Parthenium* sp. was used as a feedstock for fermentable sugar production after pretreatment with mild alkali (1% NaOH). An attempt was also made to carry out monoculture and co-culture fermentation of enzymatic hydrolysates of pretreated biomass using *S. cerevisiae* and *P. stipitis* to compare the efficiency of these yeasts for fermentation of sugars to ethanol.

### 6.2 Materials and methods

#### 6.2.1 Raw material and chemicals

*Parthenium* sp. biomass, collected from farms of Indian Agricultural Research Institute, New Delhi, India, was air dried and chopped into a particle size of 1 cm and stored in an airtight polythene bag until further use.

Accellerase® 1500, a commercial cellulase enzyme complex from *Trichoderma reesei* (Endoglucanase: 2200-2800 CMC U/g; FPase: 520-620 FPU/g; β-glucosidase: 525-575 U/g and residual xylanase: 115 U/g) was purchased from Genencor (New York, USA). DNSA (3,5 - dinitrosalicylic acid) and ethanol were purchased from Sisco Research Laboratories Pvt. Ltd (Mumbai, India) and Merck (Darmstadt, Germany), respectively.

#### 6.2.2 Compositional analysis of *Parthenium* biomass

The *Parthenium* sp. biomass was extracted with benzene-ethanol (2:1, v/v) mixture followed by absolute ethanol. The extractive-free substrate was used for analysis of cellulose (Updegraff, 1969), pentosans, klason lignin, ash and moisture content (TAPPI, 1997).

#### 6.2.3 Mild alkali pretreatment of *Parthenium*

Alkali pretreatment was carried out by suspending 5 g of *Parthenium* sp. sample with 1% (w/v) sodium hydroxide in 250 mL Erlenmeyer flask with solid to
liquid ratio of 1:10 and kept under static condition for 1 h at room temperature (40-45° C). Pretreated substrates were filtered through muslin cloth and the solid residue was washed several times with distilled water to neutralize the pH after which they were dried at 70° C till constant weight. The dried substrates were used for enzymatic hydrolysis.

6.2.4 Enzymatic hydrolysate production and its concentration

Enzymatic hydrolysis was carried out in 250 mL Erlenmeyer flask by suspending pretreated cellulosic residue of *Parthenium* sp. with 100 mL of 50 mM sodium citrate buffer pH (4.5) in shaking water bath at 50°C, with a substrate loading of 2.4% (w/v). The pretreated substrate was soaked in sodium citrate buffer for 30 min before adding Accellerase enzyme. Sodium azide (2%) was also added to restrict any microbial contamination during course of enzymatic hydrolysis. The soaked substrate was supplemented with enzyme complex, Accellerase® 1500 (8% v/v) and the hydrolysis was carried out for 48 h with agitation of 150 rpm. Samples were withdrawn at regular intervals and centrifuged at 10000 rpm for 10 min at 4 °C. The cell free supernatants were used to analyze the total reducing sugars released by DNS method (Miller, 1959). Enzymatic hydrolysate was concentrated by rotary evaporator to a concentration of 47.34± 1.23 g/L total reducing sugar which was used for ethanol fermentation. The saccharification efficiency was calculated as:

\[
\text{Saccharification (\%)} = \frac{\text{Reducing sugars released (mg) x 0.9}}{\text{Carbohydrate content in pretreated biomass (mg)}} \times 100
\]

6.2.5 Ethanol Fermentation of enzymatic hydrolysate

6.2.5.1 Microorganisms and inoculum preparation

The yeast *Saccharomyces cerevisiae* LN1 was obtained from the culture collection by Division of Microbiology, Indian Agricultural Research Institute, New Delhi, India and *P. stipitis* NCIM 3498 was procured from National Collection of Industrial Microorganism (NCIM), National Chemical Laboratory (NCL), Pune, India. Both yeasts were maintained in MGYP (g/L: Malt extract-3g; glucose-10g; yeast extract-3g; peptone-5g; agar-20g; pH 7.8) agar slants. The inoculum was prepared by growing cells at 30 °C for 48 h on MGYP broth (pH 7.0 ± 0.2) and incubated in rotary shaker under agitation at 150 rpm.
6.2.5.2 Fermentation
The batch fermentation of enzymatic hydrolysate (47.34 g/L) was carried out in 100 mL Erlenmeyer flaks with the working volume of 20 mL and the initial pH of the hydrolysate was adjusted to 5.0 by adding NaOH (2M). The hydrolysate was inoculated with 10% (v/v) inoculum of *S. cerevisiae/P. stipitis*. In the co-fermentation experiment both the yeasts were inoculated in equal volume [5% (v/v) of each culture]. The fermentation temperature was kept constant at 30 ± 0.1 °C in an incubator under static condition for 96 h. Samples were withdrawn at intervals of 12 h and centrifuged at 10,000 rpm for 15 min at 4 °C. The cell free supernatant was used to determine the ethanol content and residual sugar concentration. The cell pellet weight was also determined.

6.2.5.3 Analytical methods
The hydrolysate was analyzed for ethanol and sugars using Waters 515 HPLC (Waters Corporation, Milford, MA, USA) equipped with a Waters 2414 refractive index detector (RID). The Aminex HPX-87H column was operated with 5 mM H₂SO₄ as an mobile phase at a flow rate of 0.5 mL/min and the oven temperature was kept at 40 °C. The ethanol standards were prepared using commercial grade ethanol (Merck, Darmstadt, Germany) and the concentration of ethanol was determined by using a calibration curve obtained from standard ethanol solutions. The dry biomass of yeast cells was determined by gravimetric method by drying the yeast pellets at 70 °C till constant weight. Total reducing sugars were estimated by DNSA method (Miller, 1959).

6.3 Results and discussion
6.3.1 Chemical composition of *Parthenium* sp. biomass
The pretreated *Parthenium* sp. biomass contained 60.2% holocellulose (40.2% cellulose and 20.0% pentosans), 16.1% kelson lignin, 6.1% ash and 6.5% moisture content. The presence of high concentration of holocellulose represents the potentiality of *Parthenium* sp. biomass as a renewable source for bioethanol production.

6.3.2 Mild alkali pretreatment
The delignification of lignocellulosic biomass, pretreated with sodium hydroxide, is mainly due to breakage of the ester bonds cross-linkage between lignin
and hemicellulose, which leads to increase in the porosity of biomass (Tarkov and Feist, 1969). The alkali treated Parthenium resulted in 16.6% reduction in lignin content along with increase in cellulose and pentosans by 30.5 and 22%, respectively. Alkaline reagents such as lime and ammonia have been reported to be effective at lower temperature i.e. temperature below 50 °C (Kim and Holtzapple, 2005; Kim and Lee, 2005a). In earlier studies, a maximum removal of lignin (19%) was observed at 23 °C in spruce fiber at 7% sodium hydroxide (Zhao et al., 2008b) and 20.1% delignification at 95 °C in corn stover pretreated with sodium hydroxide (0.1 g/g dry biomass). Although pretreatment at high alkali loading, temperature and longer residence time can increase delignification and digestibility of substrates, the high severity conditions may also lead to sugar loss through dissolution and degradation of hemicellulose. Hence, a balance between extent of delignification and carbohydrate preservation has to be established in order to achieve maximum sugar yield (Chen et al., 2013).

6.3.3 Enzymatic hydrolysis of pretreated Parthenium sp.

During the time course of saccharification of delignified cellulosic substrate, a perpetual increase in sugar release was observed. The maximum sugar yield (577.5 mg/gds) was achieved after 48 h of incubation with the saccharification rate of 12.03 mg/g/h. The maximum saccharification rate (17.84 mg/g/h) was achieved after 16 h of incubation which started declining thereafter. It could be due to the increase in the substrate resistance (Gupta et al., 2009) or end product inhibition of the enzymes by glucose and cellobiose (Kuhad et al., 1999). Moreover, 86.3% saccharification efficiency was obtained which is in accordance with previous results reported in earlier studies with a saccharification efficiency of 82% and 86.5% in alkali treated rice straw (Ioelovich and Morag, 2012) and switch grass (Wang et al., 2012), respectively.

6.3.4 Fermentation

The enzymatic hydrolysate containing 47.34± 1.23 g/L sugars when fermented with S. cerevisiae LN1 produced 12.89± 0.23 g/L ethanol (ethanol yield 0.27 g/g and productivity of 0.36 g/L/h at 36 h) with 0.08 g/g biomass yield (Table 1). The ethanol yield by S. cerevisiae LN1 (0.27 g/g) was similar to the earlier reports of ethanol yield of 0.3 g/g from rice straw (Yadav et al., 2011) and 0.23 g/g from water hyacinth (Singh and Bishnoi, 2013) at 36 h of incubation. Among
Table 1
Fermentation profile of enzymatic hydrolysate with *Saccharomyces cerevisiae* LN 1

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Ethanol (g/L)</th>
<th>Sugar (g/L)</th>
<th>Ethanol yield (g/g)</th>
<th>Ethanol productivity (g/l/h)</th>
<th>Biomass yield (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>47.34± 1.23</td>
<td>-</td>
<td>-</td>
<td>0.02</td>
</tr>
<tr>
<td>12</td>
<td>4.91 ± 0.04</td>
<td>35.23± 2.37</td>
<td>0.10</td>
<td>0.41</td>
<td>0.05</td>
</tr>
<tr>
<td>24</td>
<td>12.43± 0.12</td>
<td>9.78± 0.11</td>
<td>0.26</td>
<td>0.52</td>
<td>0.06</td>
</tr>
<tr>
<td>36</td>
<td>12.89± 0.23</td>
<td>8.02± 0.09</td>
<td>0.27</td>
<td>0.36</td>
<td>0.08</td>
</tr>
<tr>
<td>48</td>
<td>12.48± 0.18</td>
<td>7.43± 0.07</td>
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<td>0.26</td>
<td>0.09</td>
</tr>
<tr>
<td>60</td>
<td>12.43± 0.13</td>
<td>7.24± 0.01</td>
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<td>0.21</td>
<td>0.09</td>
</tr>
<tr>
<td>72</td>
<td>12.23± 0.14</td>
<td>7.12± 0.02</td>
<td>0.25</td>
<td>0.17</td>
<td>0.09</td>
</tr>
<tr>
<td>84</td>
<td>11.72± 0.09</td>
<td>6.97± 0.02</td>
<td>0.24</td>
<td>0.14</td>
<td>0.10</td>
</tr>
<tr>
<td>96</td>
<td>11.52± 0.10</td>
<td>6.94± 0.01</td>
<td>0.24</td>
<td>0.12</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Data represents mean ± SD, *n=3*
Table 2

Fermentation profile of enzymatic hydrolysate with *P. stipitis* NCIM3498

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Ethanol (g/L)</th>
<th>Sugar (g/L)</th>
<th>Ethanol yield (g/g)</th>
<th>Ethanol productivity (g/l/h)</th>
<th>Biomass yield (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>47.34± 1.23</td>
<td>-</td>
<td>-</td>
<td>0.02</td>
</tr>
<tr>
<td>12</td>
<td>0.36± 0.02</td>
<td>43.25± 0.72</td>
<td>0.01</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>24</td>
<td>0.73±0.04</td>
<td>42.09± 0.34</td>
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<td>0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>36</td>
<td>1.17± 0.04</td>
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<td>0.05</td>
</tr>
<tr>
<td>48</td>
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<td>0.04</td>
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<tr>
<td>60</td>
<td>2.77± 0.07</td>
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</tr>
<tr>
<td>72</td>
<td>10.98± 0.28</td>
<td>8.22± 0.06</td>
<td>0.23</td>
<td>0.15</td>
<td>0.17</td>
</tr>
<tr>
<td>84</td>
<td>10.76± 0.19</td>
<td>7.89± 0.02</td>
<td>0.22</td>
<td>0.13</td>
<td>0.18</td>
</tr>
<tr>
<td>96</td>
<td>10.38± 0.14</td>
<td>6.93± 0.03</td>
<td>0.22</td>
<td>0.11</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Data represents mean ± SD, n=3
Table 3

Co-fermentation profile of enzymatic hydrolysate with both *Saccharomyces cerevisiae* LN 1 and *Pichiastipitis* NCIM 3498

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Ethanol (g/L)</th>
<th>Sugar (g/L)</th>
<th>Ethanol yield (g/g)</th>
<th>Ethanol productivity (g/l/h)</th>
<th>Biomass yield (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>47.34 ± 1.23</td>
<td>-</td>
<td>-</td>
<td>0.02</td>
</tr>
<tr>
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<td>0.84 ± 0.02</td>
<td>44.41 ± 0.84</td>
<td>0.02</td>
<td>0.07</td>
<td>0.05</td>
</tr>
<tr>
<td>24</td>
<td>13.78 ± 0.15</td>
<td>8.79 ± 0.18</td>
<td>0.29</td>
<td>0.57</td>
<td>0.07</td>
</tr>
<tr>
<td>36</td>
<td>14.28 ± 0.31</td>
<td>7.57 ± 0.11</td>
<td>0.30</td>
<td>0.40</td>
<td>0.08</td>
</tr>
<tr>
<td>48</td>
<td>13.60 ± 0.20</td>
<td>7.42 ± 0.08</td>
<td>0.28</td>
<td>0.28</td>
<td>0.09</td>
</tr>
<tr>
<td>60</td>
<td>13.54 ± 0.14</td>
<td>7.30 ± 0.04</td>
<td>0.28</td>
<td>0.23</td>
<td>0.10</td>
</tr>
<tr>
<td>72</td>
<td>13.45 ± 0.09</td>
<td>7.21 ± 0.03</td>
<td>0.28</td>
<td>0.19</td>
<td>0.11</td>
</tr>
<tr>
<td>84</td>
<td>12.92 ± 0.11</td>
<td>6.88 ± 0.01</td>
<td>0.27</td>
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<td>0.12</td>
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<tr>
<td>96</td>
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<td>6.82 ± 0.01</td>
<td>0.26</td>
<td>0.13</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Data represents mean ± SD, *n* = 3
different pentose fermenting yeasts, *P. stipitis* has shown high potential as it has broad substrate specificity and no requirement of vitamins for pentose utilization (du Preez et al., 1986). Fermentation with *P. stipitis* NCIM3498 gave 10.98 ± 0.28 g/L ethanol (ethanol yield 0.23 g/g and productivity 0.15 g/L/h at 72 h) with 0.17 g/g biomass yield (Table 2). Similar result was reported by Chandel et al. (2011) from *Saccharum spontaneum* pretreated with sodium hydroxide and fermented by *P. stipitis* NCIM3498 (ethanol production 17.44 g/L, yield 0.39 g/g and productivity 0.24 g/L/h after 72 h). The ethanol yield of 0.23 g/g by *P. stipitis* was in agreement with previous reports of ethanol yield 0.35 g/g from water hyacinth (Nigam, 2001), 0.36 g/g (Kapoor et al., 2008) and 0.33 g/g from *Lantana camara* (Kuhad et al., 2010).

Co-culture fermentation with both *S. cerevisiae* LN1 and *P. stipitis* NCIM 3498 resulting in maximum production of ethanol (14.28± 0.31 g/L) as well as maximum ethanol yield (0.30 g/g) and productivity (0.4 g/L/h) at 36 h and declined slowly after that (Table 3). Similar observation was made in previous study by Yadav et al. (2011) in rice straw using co-culture of *S. cerevisiae* (OVB 11) and *P. stipitis* NCIM3498 resulted in maximum production of ethanol (12 g/L) and ethanol yield (0.40 g/g) at 36 h. Singh and Bishnoi (2013) also reported in water hyacinth hydrolysate by using co-culture of *S. cerevisiae* and *Scheffersomycesstipitis* which resulted in maximum production of ethanol (9.8 g/L) with ethanol yield of 0.33 g/g at 36 h. The yield and productivity of ethanol obtained from co-culture were higher than those in a single culture. The increase in ethanol yield was not only based on the better fermentation efficiency of *S. cerevisiae* compared to that of *P. stipitis* but also due to xylose fermentation by *P. stipitis*. This could be due to less oxygen consumption of the *S. cerevisiae* in the co-culture which favoured the fermentation of *P. stipitis* (Taniguchi et al., 1997) in co-culture.

6.4 Conclusion

This study showed the efficiency of alkali pretreatment due to significant removal of lignin and maximum release of sugars after enzymatic hydrolysis of *Parthenium* sp. The co-fermentation of enzymatic hydrolysates with *S. cerevisiae* LN1 and *P. stipitis* NCIM3498 resulted in maximum ethanol production as well as ethanol yield than monoculture fermentation of these yeasts. The results clearly demonstrate that *Parthenium* sp. represents as a cheaper, renewable and sustainable
feedstock for ethanol production. Further studies needs to be carried out to optimize the fermentation process to achieve the maximum production of ethanol.

Acknowledgements

The authors are thankful to Post Graduate School and Director, Indian Agricultural Research Institute, New Delhi for providing fellowship towards Ph.D. programme and National Fund for Basic, Strategic and Frontier Application Research in Agriculture (ICAR).
7. DISCUSSION

Lignocellulosic biomass has been projected to be one of the main resources for economically attractive bioethanol production. Although theoretical ethanol yields from sugar and starch are higher than from lignocellulose, these conventional sources are insufficient for worldwide bioethanol production. In this context, agricultural residues and weedy biomasses are renewable, low cost and abundantly available resources and can substitute the sugar and starch substrates. The huge biomass availability of agricultural residues makes it as potential substrate for bioethanol production but these substrates also being used as feed and fodder. Perennial herbaceous weed biomass can serve as good feedstocks as they do not require annual reseeding once established, need fewer energy inputs such as fertilizer and pesticides than annual crops (Dien et al., 2005). These weeds also do not demand separate land and have no feed value as well.

In lignocellulosic biomass, the presence of lignin and hemicelluloses obstructs the access of cellulase enzymes to cellulose (Mansfield et al., 1999), thus reducing the efficiency of the hydrolysis. Lignin limits the rate of enzymatic hydrolysis by acting as a physical barrier, preventing the digestible parts of the cellulose and reduce its hydrolysis by non productively binding to cellulolytic enzymes (Chang and Holtzapple, 2000). Removal of hemicelluloses increases the mean pore size of the substrate and therefore increases the accessibility of cellulose for enzymatic hydrolysis (Chandra et al., 2007). Pretreatment of lignocellulosics aims to decrease crystallinity of cellulose, increase biomass surface area, remove hemicellulose, and break the lignin barrier. Pretreatment makes cellulose more accessible to hydrolytic enzymes to facilitate conversion of carbohydrate polymers into fermentable sugars in a rapid way with the concomitant higher sugar yields (Alvira et al., 2010).

Different pretreatments have been shown to be better suited for specific feedstocks e.g. alkaline based pretreatment methods can effectively reduce the lignin content of agricultural residues (Chandra et al., 2007), acid pretreatment have been shown to be effective on wide range of lignocellulosic substrates, but relatively expensive (Moiser et al., 2005b). Since different lignocellulosic materials have different physico-chemical characteristics, it is necessary to adopt suitable pretreatments based on their properties. The pretreatment step has been recognized
as a technological bottleneck for the cost-effective development of bioprocesses from lignocellulosic materials, also it has a large impact on all subsequent steps in the overall conversion process in terms of cellulose digestibility, generation of toxic compounds and energy demands (Galbe and Zacchi, 2007). In this work we analyze the potential of several pretreatment methods for bioethanol production from weedy lignocellulosic biomass.

Many pretreatment methods have been used for conversion of lignocellulosic biomass into ethanol. In chemical method, acid pretreatment targets hemicelluloses whereas alkali-catalyzed pretreatment mainly used for removal of lignin (Hahn-Hägerdal et al., 2006). In biopretreatment, lignolytic organisms colonise substrate and preferentially degrade lignin to gain access to cellulose and hemicelluloses. Lignocellulolytic microorganisms, especially fungi, have created interest as biomass degraders for large scale biotreatment applications due to their ability to produce high amounts of extracellular lignocellulolytic enzymes. *P. chrysosporium*, a white rot fungus, was successfully used for biological pretreatment of cotton stalks by solid state fermentation which facilitated the conversion of substrate to ethanol (Shi et al., 2008). Delignification of paddy straw with the white rot fungus, *Trametes hirsuta* (Saritha et al., 2012) and a micromycete, *Myrothecium roridum* LG7 (Tiwari et al., 2013) under solid state fermentation enhanced the recovery of sugars during enzymatic saccharification of paddy straw. A comparison of pretreatment methods to assess the enzymatic accessibility and hydrolysis of pretreated lignocellulosic substrates revealed that biological pretreatment is an effective method for facilitating the enzymatic hydrolysis (Chandra et al., 2009).

Various sugars (pentose, hexose, and oligosaccharides) are derived from acid/enzymatic hydrolysis of pretreated lignocellulosic biomass. *S. cerevisiae* and *Z. mobilis* are the best known alcohol-fermenting organisms capable of fermenting only hexose sugars and sucrose into ethanol. But pentose fermenting microorganisms such as *P. stipitis*, *C. shehatae*, and *P. tannophilus* can produce ethanol from a variety of sugar substrates (Hahn-Hägerdal et al., 2007). In the case of pentose and hexose sugar mixture, a co-culture system consisting a potential glucose and xylose-fermenting yeasts will be useful (Taniguchi et al., 1997) for enhanced ethanol production.
7.1 Bioprospecting of microbial culture for biological delignification of *Parthenium* biomass

In the present research work, twenty eight fungal strains were isolated and screened qualitatively and quantitatively for their lignocellulolytic enzyme activities by using *Parthenium* sp. biomass as a sole carbon source. The main aim of this experiment was to select a potent lignolytic fungus with minimum cellulolytic activity. In qualitative assay, all the twenty eight isolates had shown clear hydrolytic zone when stained with congo red solution followed by NaCl (1M) on RMM agar plates with 1% CMC as a carbon source. This indicates that all the isolates were capable of producing cellulolytic enzymes. Presence of lignolytic enzymes was detected only in four isolates. These cultures had shown bright red zone around the fungal disc which indicates the production of laccase and lignin peroxidase due to oxidation of guaiacol into quinone by the action of the above mentioned enzymes (Thorn et al., 1996). In quantitative assay, all the isolates produced cellulolytic enzymes in a variable range. The isolate PK-18 had shown higher CMCase and FPase activity. Isolate PK-16 and PK-23 produced more β-glucosidase and xylanase enzymes, respectively. Based on sequencing of ITS region of selected fungal isolates, PK-16, PK-18 and PK-23 were identified as *Aspergillus niger*, *Aspergillus flavus* and *Trichoderma longibrachiatum*. The isolate PK-27, identified as *Marasmiellus palmivorus*, was found to be the most promising isolate as it had shown high lignolytic activity with low level of cellulolytic enzymes which favoured its selection for biopretreatment of *Parthenium* by solid state fermentation (SSF). The lignolytic enzyme production (69.1 IUml⁻¹) by *M. palmivorus* PK-27 was comparable with other white rot fungi *Trametes hirsuta* used in earlier studies producing 42.08 IUml⁻¹ (Rana et al., 2013) and 57.9 IUml⁻¹(Saritha et al., 2012) ligninase with *Parthenium* and paddy straw as substrate, respectively.

7.2. Biological and chemical pretreatment of *Parthenium* biomass

An important aspect of biological pretreatment is that the lignolytic fungi should involve least loss of carbohydrate content. Microorganisms with high lignolytic activity and low cellulolytic activity are important for conservation of holocellulose and maximization of sugar recovery which determines the efficiency of biological pretreatment (Shi et al., 2008). In the present study, SSF was carried out with selected lignocellulolytic fungus *M. palmivorus* PK-27 to evaluate its
potential for lignin degradation in *Parthenium* biomass. Lignin degrading white rot fungi reported to be the most suitable and effective for biological pretreatment of lignocellulosic materials as the degradation occurs through the action of laccases and lignin peroxidases (Kumar et al., 2009a).

Chemical pretreatment i.e. 1% (v/v) sulphuric acid and 1% (w/v) NaOH was also employed for delignification of *Parthenium* sp. as these treatments had also been reported to achieve better enzymatic saccharification as compared to untreated sample (Kapoor et al., 2008). All these methods resulted in significant removal of lignin. The treated *Parthenium* biomass was also subjected to saccharification process.

The enzymatic hydrolysis with Accellerase®1500, a potent cellulase cocktail specifically prepared for hydrolysis of pretreated lignocellulosics for bioethanol production, resulted in better saccharification and maximum amount of reducing sugars (513.1 ± 40.1 mggd⁻¹) with the saccharification yield of 76.6% was observed in alkali pretreated substrate as compared to other two methods. Similar result had been reported by Chen et al. (2009) who compared four different chemical pretreatments for enhancing enzymatic digestibility of corn stover. Among all the methods tested, the hydrolysis yield of NaOH pretreated corn stover was 81.2% within 48 h and the enzymatic hydrolysates contained higher content of total reducing sugars and less inhibitors, which is suitable for subsequent fermentation process to produce ethanol. Similarly, Chandel et al. (2009b) had also reported enhanced saccharification and high sugar recovery during enzymatic hydrolysis of alkali pretreated *S. spontaneum*. It had been reported that cellulose hydrolysis improved with higher removal by reducing non-specific adsorption of cellulase enzyme (Yang and Wyman, 2004). Furthermore, the chemical modification of functional groups in lignin, which resulted in the conversion of lignin to hydroxypropylated lignin, resulted in the transformation of lignocellulose into a more favourable form for cellulosic hydrolysis (Sewalt et al., 1997). All these studies proved that lignin removal plays a crucial role in enhancing enzymatic digestibility of treated substrate.

In our study, the comparatively reduced amount of total reducing sugars (476 ± 30.2 mggd⁻¹) in acid pretreated substrates than alkali treatment, could be due to solubilisation of hemicelluloses as well as generation of toxic compounds (Alvira et al., 2010). This treatment also had less influence on lignin, since negligible amount
of lignin was recovered from the washed extractive. In dilute acid hydrolysis, the hemicelluloses fraction is depolymerised at lower temperature than the cellulose fraction. But at higher temperature or longer retention times, the released monosaccharides are further degraded (Yadav et al., 2011) to other compounds such as furfural and hydroxy methyl furfural (HMF) which affect metabolism of microorganism during fermentation step (Saha et al., 2005). Furthermore, the condensed lignin remains on the surface of crystalline cellulose following dilute acid pretreatment, which can potentially block enzyme accessibility in the substrate during saccharification, resulting in less sugar yield (Liu and Wyman, 2004).

The neutralization of chemically pretreated lignocellulose without subsequent water washing was found to result in decreased glucose yield from hydrolysis of the pretreated lignocellulose when compared to hydrolysis of pretreated lignocelluloses that was washed. This is probably because of the presence of inhibitory substances formed during acid or alkali pretreatment under severe conditions. HMF and furfural mainly formed during acid pretreatment and carboxylic acids such as acetic acid and levulinic acid that are primarily formed during alkali pretreatment could also be inhibitory to enzymatic hydrolysis (Merino and Cherry, 2007).

In this study, biopretreated substrates produced 410 ± 45.1 mggd⁻¹ total reducing sugars after 48 h of enzymatic hydrolysis. The results are in accordance with the earlier studies of Gupta et al. (2011) carried out with Pycnoporus cinnabarianus pretreated P. juliflora and L. camara under solid state fermentation which resulted in 402.1 and 389.1 mggd⁻¹ sugars respectively after 48 h of enzymatic hydrolysis with cellulases. The increased sugar yield compared to untreated substrates may be attributed to the partial degradation of lignin seal which was responsible for preventing the penetration of cellulase molecules during enzymatic hydrolysis (Taniguchi et al., 2005). Moreover, the improvement in enzymatic hydrolysis could be because the biological pretreatment increased the initial adsorption of cellulases to the cellulose, which in turn enhanced the level of released sugars during saccharification (Yu et al., 2009). Although the biologically pretreated substrates produced significant amount of reducing sugars these are comparably less than chemically pretreated substrates. However, when compared to chemical pretreated substrates, fungal pretreated substrates can be used directly for the enzymatic hydrolysis with one washing. Therefore, even though fungal pretreatment produces a product with lower enzymatic digestibility than chemical
pretreatment, fungal fermentation of lignocellulose can lead to reduced operating costs for washing and the removal of inhibitors (Bak et al., 2010).

The pretreated *Parthenium* biomass was also subjected to Scanning electron microscopy (SEM), X-ray diffraction (XRD), and Fourier transform infrared spectroscopy (FTIR) analysis to understand the structural and chemical changes during different pretreatment processes. Scanning electron microscopy (SEM) images of untreated substrate displayed a regular compact and smooth surface, indicating a highly ordered surface structure while pretreated substrates reveal formation of some holes on the biomass surface and disruption of the biomass network consistent with hemicelluloses and lignin removal during pretreatment (Kim and Lee, 2005a). Dilute acid treatment reduced the fibre length and totally disrupted the biomass structure. Alkali treatment resulted in formation of many apertures of various sizes on the surface. The removal of lignin and partial degradation of hemicelluloses are likely to be important factors in increasing the enzymatic digestibility of pretreated substrates. Saini et al. (2013) reported that the surface layer was removed and broken or made loose during pretreatment resulting in exposure of internal structure and fibres. It seems that exposing the cellulose through either structural modification or alteration of lignocellulosic structure is a key factor in enzymatic hydrolysis of the remaining carbohydrates fraction (Chandel et al., 2011) in pretreated *Parthenium* biomass.

Among the several features, crystallinity index is believed to be a significant factor which affects enzymatic saccharification of glucan (Mansfield et al., 1999). Lignin and hemicellulose in lignocellulosic biomass are considered to be amorphous components, while cellulose is considered to be the crystalline component. Pretreatment of lignocellulosic biomass is to disrupt the lignin-carbohydrate complex, to decrease native cellulose crystallinity and to partially remove lignin and hemicelluloses, had been shown to significantly enhance the subsequent hydrolysis of cellulose (Moiser et al., 2005; Kumar et al., 2009). The CrI of lignocellulose is negatively proportional to the extent of hydrolysis of cellulose (Gharpuray et al., 1983). The increased crystallinity in acid (44.5%) and alkali (39.7%) pretreated substrate was observed in this study. This increase in CrI in acid treated substrate is consistent with the results reported by Kumar et al. (2009) and Li et al. (2010) suggesting that the amorphous cellulose breaks down more under acidic condition but this pretreatment is unable to break apart the inter and intra hydrogen bonds in
cellulose fibrils (Li et al., 2010). In alkali pretreated substrate, the increase in crystallinity index may be attributed to increased hydrolyzation and peeling reaction in the amorphous region than the crystalline region (He et al., 2008). Sodium hydroxide can destroy cellulosic crystalline areas and enlarge the pore ratio and inner surface areas which is advantageous during enzymatic hydrolysis (He et al., 2008). As the lignin and xylan are both amorphous, their removal increased the crystallinity index, however the drop in lignin during alkali pretreatment and reduction of hemicelluloses and lignin content after acid pretreatment improved enzyme access to cellulose thus enhancing cellulose digestion by enzymes despite an increase in crystallinity index (Kumar and Wyman, 2009). In the biological pretreated sample, reduction in CrI was observed which may be attributed to the breakdown of inter/intra hydrogen bonds in crystalline portion of cellulose. Similar results were reported by Tiwari et al. (2013) and Rana et al. (2013) suggesting that the loss of inter- and intra-molecular hydrogen bonding can lead to some new crystal structure in the biologically pretreated substrates.

The FTIR spectroscopy is extensively used for lignocellulose characterization since it presents a relatively easy and non-destructive method of obtaining direct information regarding chemical changes that occurs during various pretreatments (Ristolainen et al., 2002). In FTIR studies, all the pretreated substrates have shown reduction in the peaks corresponding to subunits and functional groups of lignin molecules and cellulose fibrils. The peak at 2900 cm$^{-1}$ is attributed to C-H stretching, and the pretreated substrates produced the greatest change indicating that the methyl and methylene portions of cellulose were ruptured. Similar observation was made in earlier studies (Kumar et al., 2009; He et al., 2008). Acid pretreated substrate had the highest reduction in the 1720 cm$^{-1}$ attributed to hemicelluloses acetyl and uronic ester groups or linkages in lignin and/or ester hemicelluloses, furilic and $p$-coumaric acid carboxylic groups (Sun et al., 2005), also the peak at 900 cm$^{-1}$ is attributed to amorphous portion of cellulososes and reduction in this peak during acid treatment might be due to high solubilisation of cellulose and hemicellulose (Kumar et al., 2009). Acid and alkali pretreatment had the greatest reduction in the 1595 cm$^{-1}$ peak assigned to aromatic skeletal vibration, and reduction or shift in this position is attributed to condensation reactions and/or splitting of lignin aliphatic side chains (Sun et al., 2005). However, alkali treatment can also release some cellulose and hemicelluloses by hydrolyzing ester bonds between lignin and cellulose or
hemicelluloses which ultimately improve the biodegradability of pretreated substrates (Nathalie et al., 2003). The results showed that the spectra of lignocellulose of untreated Parthenium had shown similar profiles but different intensities of the absorption bands. The differences indicated that the structure of cellulose and lignin was changed after treatment and the changes were both intramolecular.

### 7.3. Enzymatic saccharification of alkali pretreated Parthenium biomass

After alkali pretreatment, the treated substrate was utilized for saccharification using the commercial cellulase preparation Accellerase®1500. Since, substrate and enzyme loading, pH and temperature are the important process parameters during enzymatic hydrolysis of substrate, optimization of conditions is necessary to improve the sugar yield by saccharification. Response surface methodology (RSM) is a statistical technique for the optimization of multiple variables, which determines optimum process conditions by combining experimental designs with interpolation by first- or second-order polynomial equations in a sequential testing procedure. Since, comparatively high release of reducing sugars was obtained from alkali pretreated Parthenium sp. as compared to other two methods employed only chemical pretreated substrate was used to optimize the variables temperature, pH, enzyme loading and substrate loading using RSM. The $R^2$ value of model (0.96) was in good and reasonable agreement with the adjusted $R^2$ (0.93) and predicted $R^2$ (0.78) value. From the ANOVA table, it was inferred that the model was statistically significant. The interaction among the variables had no significant influence except temperature and substrate loading on saccharification yield.

Response surface plot provides a method to predict the degree of saccharification for different values of the test variables. The three-dimensional response surface was plotted between two variables while other variables were held at zero level. Saccharification yield was decreased as the temperature increased beyond 55°C during enzymatic hydrolysis. Similar observation was made by Karki et al. (2011) with Accellerase®1000 enzyme complex. Jeya et al. (2009) and Ferreira et al. (2009) also reported that at higher temperature, the saccharification efficiency was decreased. This could be due to thermal inactivation of enzymes during saccharification. The optimum temperature for maximum saccharification yield was
50°C which is in accordance with the result of Saini et al. (2013), Jagtap et al. (2012).

The increase in enzyme loading resulted in enhanced saccharification yield may be attributed to increase in the ratio of substrate to enzyme (Huang and Penner, 1999). There is certain limit for enzyme concentration after which higher enzyme loadings have no significant influence on the product formation. It has also been reported that substrates having a good pretreatment usually require lower enzyme concentrations than those with poorer pretreatments (Chambel, 2008). The adsorption of enzymes to substrate, saturation of cellulose surfaces with enzymes, transformation of cellulose to less digestible form and inhibition of enzyme activity due to accumulation of hydrolysis products (Lee and Fan, 1982; Soto et al., 1984) are the possible reason for negligible influence of enzyme loading on saccharification yield. The adsorption of Accellerase to the lignin molecules is also possibly responsible for the need of higher enzyme loadings to obtain higher glucose yield (Kumar and Wyman, 2009).

The increase in substrate loading resulted in reversal of response and it could be due to the difficulties in mixing by reducing the aqueous phase in the mixture and thereby hindered enzymatic hydrolysis (Xu et al., 2007). Further increase in substrate loading results in constant rate of product formation. Similar observation was reported by Jeya et al. (2009) in alkali pretreated rice straw and the optimum substrate loading for maximum saccharification yield of 88% was achieved with the substrate loading of 2.5%. Our result of 2.4% substrate concentration is in accordance with this study. Substrate loading is considered to be one of the major factors affecting the conversion rate of enzymatic hydrolysis of cellulose (Qi et al., 2009). Based on the previous studies, it was observed that maximum saccharification yield was achieved at a substrate concentration of below 10% (w/v).

The pH had no significant influence on saccharification yield. But substrate loading and enzyme loading were interdependent affecting the saccharification yield. From the above results, it can be inferred that the temperature (50°C) and pH (4.53), high enzyme loading (8%) and substrate loading (2.4% w/v) resulted in increased saccharification yield. From an economic point of view, it is desirable to have the lowest possible enzyme concentration and highest possible substrate loading to achieve the desirable saccharification rate (Jeya et al., 2009). Most of these conditions were achieved in the present study.
The predicted value of 80.08% saccharification yield by the model was validated by experimental value (85.80%) indicating that both were in good agreement and showed that the model is useful for predicting the optimal conditions for variables which influence the saccharification yield. In general, optimization with RSM enhances experimental results more than the conventional optimization methods (Saini et al., 2013). In the earlier studies, the saccharification yield was enhanced to 51% through optimization, which was higher than the conversion yield of 32% from rice straw (Saha and Cotta, 2008) and 41% from sugarcane bagasse (Kaar et al., 1998).

This optimization analysis revealed the optimum condition of 50°C, pH 4.53, enzyme loading of 8% and substrate loading of 2.4% for saccharification of chemically pretreated *Parthenium* biomass. This also identified that the temperature, enzyme concentration and substrates loading are significant factors in the saccharification process. The higher saccharification yields were reported in the region of moderate-to-higher enzyme loadings and in the temperature range of 50-55°C, the substrate loading varied from 1% to 3%, indicating that the substrate loading are more crucial than enzyme loading/temperature in the saccharification process. The optimum process parameters along with mild alkali pretreatment at room temperature (40-45°C) has additional advantage of producing a clean substrate which is highly digestible and rich in cellulose and pentosans (Chen et al., 2013). In addition to this, it also provides a probable reduction in cost of saccharification with minimum energy requirement. The increased amount of total reducing sugars will be advantageous as the ethanol production can be enhanced by increased availability of fermentable sugars.

**7.4. Ethanolic fermentation of saccharified *Parthenium* biomass hydrolysate**

The saccharification process was carried out under optimum conditions to obtain the enzymatic hydrolysates for ethanolic fermentation. The hydrolysate was concentrated to get a 4-fold increase in fermentable sugars. The hydrolysate was vacuum evaporated with the main purpose of increasing the sugar content to facilitate high ethanol yield during fermentation. The concentrated sugar hydrolysate was fermented with *S. cerevisiae* and *P. stipitis* for monoculture fermentation. In co-culture fermentation equal volume of both yeasts was inoculated and incubated for 96 h at 30°C.
The *S. cerevisiae* produced a maximum of 12.89 ± 0.23 g/L ethanol with ethanol yield of 0.27 g/g. It produced maximum ethanol yield after 36 h and the ethanol yield declined up to 96 h. Fermentation with *P. stipitis* resulted in ethanol concentration of 10.98 ± 0.28 g/L and ethanol yield 0.23 g/g after 72 h which declined thereafter. The bioprospecting of xylose-fermenting yeasts has been area of interest in the microbial conversion of lignocellulosic hydrolysates to ethanol because almost all the hexose and pentose sugars released from both cellulose and hemicelluloses could then be converted to ethanol (Taniguchi et al., 1997). *P. stipitis* has the ability to utilize nearly all fractions of sugars present in lignocellulose hydrolysate, which is essential for the success of biorefinery (Chandel et al., 2011). The rate of fermentation of glucose to ethanol by *S. cerevisiae* is higher than that by *P. stipitis*. Two stage fermentation was carried out by Taniguchi et al. (1997) in which glucose was converted to ethanol within 12 h by *S. cerevisiae*. However, in the second stage, xylose was consumed at a rather low rate, and only a very small amount of ethanol was produced by *P. stipitis*. The conditions of the second stage of the culture were unfavourable for ethanol production probably due to the consumption of oxygen by *S. cerevisiae*.

In this study, however, the co-culture fermentation resulted in maximum ethanol concentration (14.28 ± 0.31 g/L) and ethanol yield (0.30 g/g) than the monoculture fermentation after 36 h. Fu et al. (2009) carried out fermentation in bioreactor as well as in the shake flask with *Z. mobilis* and *P. stipitis*. They observed that *Z. mobilis* inhibited the xylose fermentation. A possible explanation for that could be oxygen deprivation or *P. stipitis* by *Z. mobilis* cells. Fu et al. (2008) reported that the growth of *Pachysolen tannophilus* in the co-fermentation with *Z. mobilis* was inhibited, resulting in the slower ethanol production and sugar consumption. This could be due to ethanol produced by *Z. mobilis* resulting in the inhibition of cell growth of *P. tannophilus*. However, in the co-fermentation, overall an ethanol yield was increased suggesting that the co-fermentation process had potential for the conversion of the sugar mixture to ethanol. The results of our study were also supported by Sornvoraweat et al. (2009) who reported that co-culture of *S. cerevisiae* and *Candida tropicalis* produced maximum ethanol in comparison to monoculture of *S. cerevisiae* using acid hydrolysate of cassava peels.

Qian et al. (2006) studied ethanol fermentation of detoxified hydrolysate by adapted co-culture *S. cerevisiae* and *P. tannophilus* which generated an exceptionally
high ethanol yield after 48 h of incubation. Yadav et al. (2011) also observed similar results when the acid hydrolysates of rice straw was fermented with co-culture of *S. cerevisiae* and *P. stipitis* resulting in more (12 g/L) ethanol production than with monoculture of *S. cerevisiae*. The increased ethanol yield and fermentation efficiency is due to the total conversion of both sugars i.e. glucose and xylose. Bari et al. (2004) and Taniguchi et al. (1997) have also reported high yields of ethanol (0.39 and 0.5 g/g) with a co-culture fermentation system consisting of *S. cerevisiae* and *P. stipitis*. The reduction in ethanol concentration after 36 h could be due to simultaneous consumption of sugar and ethanol by the microbial cells at stationary phase (Ramon-Portugal et al., 2004). The increase in yeast biomass resulted from utilization of sugars present in the hydrolysate since multiplication of cell was accompanied by fermentation/ metabolic activities.

The co-culture fermentation offers an advantage of high ethanol production in addition to complete utilization of fermentable sugars i.e. glucose and xylose. There is a need to further optimize the co-fermentation system, relief from ethanol inhibition and the manipulation of oxygen levels for the significant fermentation of xylose to ethanol for the improvement in ethanol yield.

In conclusion, the mild alkali pretreatment method seems to be the promising process to improve the saccharification efficiency of the *Parthenium* biomass resulting in higher release of total reducing sugars. This study processes the promise of *Parthenium* biomass as a potential feedstock for bioethanol production in near future. Based upon area (35 m ha) infested by *Parthenium* weed and its biomass production potential (4 t ha⁻¹), 14 million litres of ethanol can be produced annually from this weed by employing the process developed in this study.
8. SUMMARY AND CONCLUSION

Lignocellulosic biomass has attracted great interest around the world for its potential as a substitute for starchy and sugary substrates to produce bioethanol. Bioethanol produced from lignocellulosic biomass is an interesting alternative since it does not compete with food crops and is less expensive as compared to conventional agricultural feedstocks. Among different lignocellulosic residues, weedy biomass would serve as a sustainable and renewable feedstock for biofuel production as its availability is all around the year. The challenge of hydrolyzing this lignocellulose into fermentable sugars is still technically problematic, as the physicochemical composition of biomass hinders the digestibility of cellulose.

In the present study, we aimed to use *Parthenium* sp. biomass as a feedstock for ethanol production. Biological and chemical pretreatments were carried out to compare the efficiency of these pretreatment processes on the release of fermentable sugars during enzymatic saccharification as well as structural and chemical changes in the biomass. The suitable pretreatment was selected and the conditions for enzymatic saccharification of pretreated substrates were optimized. The enzymatic hydrolysate was fermented further using hexose and pentose fermenting yeasts for bioethanol production.

A total of twenty eight fungal strains were isolated from various samples of degraded biomass and screened for their lignocellulolytic activity through qualitative and quantitative assay. All the isolates exhibited activities of cellulase but only four isolates (PK-6, 10, 11 and 27) possessed lignolytic activity which was revealed by chromogenic changes on screening media. The quantitative assay of all the hydrolytic enzymes was carried out under submerged fermentation with 1% (w/v) *Parthenium* as substrate for 7 days under stationary conditions. Among all the isolates, PK-18 produced higher amount of CMCase (2.92 IUml$^{-1}$) and FPase (0.22 IUml$^{-1}$) enzymes. The isolates PK-16 and PK-23 were found to show higher level of $\beta$-glucosidase (2.36 IUml$^{-1}$) and xylanase (14.75 IUml$^{-1}$) activity, respectively in submerged fermentation with 1% (w/v) *Parthenium* as substrate. The isolate PK-27 exhibited high level of lignolytic enzyme activity such as laccase (69.1 IUml$^{-1}$) and lignin peroxidase (2.83 IUml$^{-1}$) with low cellulolytic activity. This is an important trait which favoured its selection for biological pretreatment of *Parthenium* sp.
On the basis of qualitative and quantitative screening, four isolates were selected for their identification based on amplification of ITS1-5.8-ITS2 region using universal fungal primers homologous to conserved sequences. On the basis of comparison of partial sequences of isolates, based on the BLAST search and comparison with sequence available in National Centre for Biotechnology Information (NCBI) site, the isolates PK-16, PK-18, PK-23 and PK-27 were identified as *Aspergillus niger*, *Aspergillus flavus*, *Trichoderma longibrachiatum* and *Marasmiellus palmivorus*.

*Parthenium* sp. biomass was biologically pretreated with *M. palmivorus* PK-27 under solid state fermentation. The biomass was also pretreated with 1% (v/v) dilute sulphuric acid at 121°C for 20 min as well as 1% (w/v) mild sodium hydroxide at room temperature for 1 h. SEM observations revealed the alterations in the biomass surface resulting in exposure of lignocellulose complex for enzymatic action. XRD patterns of acid and alkali pretreated substrates showed an increase in crystallinity index (CrI) due to solubilisation of amorphous regions of lignocellulosic biomass. The biopretreated substrate showed reduction in CrI indicating the breakdown of hydrogen bonds in the cellulose fibrils which may lead to increased accessibility of enzymes to cellulose. From the FTIR spectra, it would be concluded that the pretreatment brought about significant alterations such as by rupturing or disruption of the functional groups of lignin/cellulose, along with partial removal of hemicelluloses.

The enzymatic saccharification of pretreated and untreated substrates with Accellerase® 1500 released a considerable amount of reducing sugars. The maximum sugar yield (513 mggds⁻¹) was achieved from alkali pretreated substrates followed by acid pretreatment (476 mggds⁻¹) and biological pretreatment (410mggds⁻¹). The reduced sugar yield from biopretreated substrates could be due to utilization of cellulose from substrates by fungal biomass during solid state fermentation, which resulted in reduced availability of holocellulose for enzymatic saccharification. On the basis of sugar release efficiency from pretreated biomass, alkali pretreatment was identified as the most suitable method for fermentable sugar production from *Parthenium* sp.

Optimization of saccharification process parameters is very important to maximize the sugar production from pretreated biomass. As the variables temperature, pH, enzyme loading and substrate loading are the most significant
factors in enzymatic saccharification, the optimization process was carried out for these variables using central composite design (CCD) in response surface methodology (RSM). The three dimensional plots were drawn by the developed model to describe the influence of independent variable and the interaction among them. Increase in temperature and substrate loading had a negative correlation with saccharification yield but increase in enzyme loading showed an increase in release of reducing sugars. The pH had no significant influence on the saccharification yield. The final saccharification experiment was carried out under optimum conditions (temperature 50°C; pH 4.53; enzyme loading 8% and substrate loading 2.4%) which resulted in high saccharification yield (85.80%) which was higher than the predicted value (80.08%) based on RSM. The reduction in enzyme loading and optimum concentration of substrate resulted in reduction in the production cost for bioethanol.

Fermentation of saccharified enzymatic hydrolysate of Parthenium sp. was carried using S. cerevisiae which produced 12.89 ± 0.23 g/L ethanol, with an ethanol yield of 0.27 g/g after 36 h of fermentation. Another pentose fermenting yeast P. stipitis resulted in 10.98 ± 0.28 g/L of ethanol with 0.23 g/g as ethanol yield after 72 h. The co-culture fermentation enhanced both the ethanol concentration (14.28 ± 0.31 g/L) and ethanol yield (0.30 g/g) within 36 h as compared with monocultures. In the hydrolysate, almost all the sugars were utilized by the inoculated yeast, since very small amount (6 g/L) of residual sugars were present in the fermentable hydrolysate. This experiment indicated that the co-culture fermentation seems to be a suitable option for fermentation, since both hexose and pentose sugars present in the saccharified lignocellulosic biomass hydrolysate were utilized by the yeasts.

These results revealed that Parthenium sp. biomass, an abundant resource may serve as a potential feedstock for bioethanol production. The most suitable approach based on the results of the present investigation is mild alkali pretreatment to loosen the biomass structure and subsequent saccharification with commercial enzyme. The enzymatic hydrolysate may then be employed for co-culture fermentation resulting in high ethanol yield. In future, better xylose-fermenting strain with higher ethanol tolerance will significantly improve the final yield of ethanol. To further optimize the co-culture fermentation system, strain(s) showing no inhibition by ethanol or concentration of sugar levels could be significant factors for further improvement of ethanol yields.
ABSTRACT

Evaluation of carrot grass (Parthenium sp.) for ethanol production

The production of bioethanol from lignocellulosic biomass, also called second generation biofuel, provides several energetic, economic and environmental advantages over conventional sources such as sugars or starch. Amongst different type of lignocellulosic substrate, weedy biomass Parthenium sp. as a feedstock for bioethanol production is less investigated. The present investigation explores its potential for bioethanol production by using chemical and biological pretreatment methods. Acid and alkali pretreatments were compared with biological pretreatment with lignolytic fungus Marasmiellus palmivorus PK-27 isolated from decaying biomass. Structural and chemical changes were examined using scanning electron microscope (SEM), fourier transform infra red (FTIR) and X-ray diffraction (XRD) analysis after chemical and biological pretreatment. Among the three pretreatment methods, alkali (1% NaOH) treatment showed significantly higher recovery of total reducing sugars (513 ± 41.0 mggd⁻¹) after 48 h of enzymatic hydrolysis with saccharification yield of 76.6% and recovery of lignin as acid precipitable polymeric lignin (APPL) (7.53 ± 0.5 mggd⁻¹). Optimization of enzymatic hydrolysis variables such as temperature, pH, enzyme loading and substrate loading rate was carried out using central composite design (CCD) in response surface methodology (RSM) to achieve the maximum saccharification yield. The experimental value of saccharification yield (85.80%) obtained was higher than the predicted value (80.08%) proposed by the developed model under optimum conditions (temperature - 50°C, pH - 4.53, enzyme loading - 8% and substrate loading - 2.4%). This illustrated a good agreement between predicted and experimental response in terms of saccharification yield. Monoculture fermentation of enzymatic hydrolysate with Saccharomyces cerevisiae LN1 resulted in ethanol yield of 0.27 g/g within 36 h while Pichiastipitis NCIM3498 produced 0.23 g/g of ethanol yield after 72 h. The co-culture fermentation with both yeasts enhanced the ethanol concentration (14.28 ± 0.31 g/L) as well as ethanol yield (0.30 g/g). These findings reveal the promise of Parthenium sp. biomass as an attractive feedstock for bioethanol production.
एथेनॉल के उत्पादनार्थ गाजर घास 
(पार्श्विनय प्रजाति) का मूल्यांकन 
सार
शर्करा अथवा स्टार्च से तैयार परमार्गत बायोइथेनॉल की तुलना में तिम्नोसेल्फूलोजिक जैव–पदार्थ से तैयार बायोइथेनॉल, तथाकथित सेंडेंड जेनेरेशन बायोइथेनॉल ऊर्जावान, कम खर्चीता एवं पर्यावरण के अधिक अनुकूल है। रासायनिक एवं जैविक पूर्व-उपचार विधियों का उपयोग कर, बायोइथेनॉल के उत्पादनार्थ एक भरण घोट के रूप में पार्श्विनय प्रजाति की व्यवस्था का अध्ययन किया गया। मुख्य रूप से अम्ल एवं क्षार पूरीपायो की तुलना से स्टार्च के जैववाद्य से विलंबित तिम्नोलाइटिक वक्र, माइक्रोएल्स पल्मीफोरस पी के–27 के साथ जैविक पूरीपायो से की गई। पूरीपायो के घसर, सेल्यूलोज के संचालक हल्के रासायनिक परिवर्तनों तथा साथ ही क्रिस्टलिनिटी का क्रम: क्रमवृत्त सीखतारी (एस ई एम), फाजरियर त्रांसफोर्म इनक्रेड (एक टी आई आर) एवं एक्स–किरण विवर्तन (एस आर डी) विलेखन दारा निरीक्षण किया गया। इन तीनों पूरीपायो विधियों में से, क्षार (1% NaOH) पूरीपायो ने एन्जायमेटिक जल–अघटन के 48 घंटों पश्चात, अम्ल अवक्षेपण योग्य पॉलीमरिक लिंगिन (ए पी पी एल) (7.53 ± 0.5 एम जी डी एस”) एवं कुल उपचारक शर्करायें (513 ± 41.0 एम जी डी एस”) के रूप में लिंगिन की महत्वपूर्ण रूप से अधिक पुनरात्मक दर्जापूर्ण, साथ ही शर्करीकरण उत्पादन 76.6% थी। अधिकतम शर्करीकरण वैद्युत्र प्राप्ति के लिए, रेस्पोंस सर्कस क्रियाविधि (आर एस एम) में सेंडेंड कंप्यूटर रिक्वायर्यान (सी सी डी) का उपयोग कर एन्जायमेटिक जल–अघटन विवर्तन यथा, तापमान, पी एच मान, एन्जायम लोडिंग एवं सूजन लोडिंग को इस्तेमाल किया गया। इस प्रकार से विकसित मोडल ने इस्तमाल अवस्थाओं (तापमान 50°सें, पी एच मान 4.53, एन्जायम लोडिंग 8% व ताप अनुपात लोडिंग 2.4%) के अन्तरमध्य 80.08% शर्करीकरण उत्पादन के पूर्वसुनिमानित मान का सुझाव दिया जिससे 85.80% के प्रायोगिक मान दारा सुस्पष्ट हुए। इससे पूर्वसुनिमानित एवं प्रायोगिक अनुक्रम के बीच अच्छा समझौता अर्थात शर्करीकरण वैद्युत की प्रशंसा होती है। नईसायग्राफिरज संचालक एल एन 1 द्वारा एन्जायमेटिक लाइसेंट के मोनोक्लार्स्न क्रिया के परिणामस्वरूप, 36 घंटों के भीतर 0.27 ग्रा./ग्रा. की इथेनॉल उत्पादन हुई जबकि पीथिया स्ट्रिप्लेक्स के साथ 72 घंटों के भीतर 0.23 ग्रा./ग्रा. इथेनॉल की प्राप्ति हुई। दोनों खिद्द के साथ सह–संबंध संबंध क्रिया से इथेनॉल–संबंध (14.28 ± 0.31 ग्रा./ली) तथा इथेनॉल उत्पाद (0.30 ग्रा./ग्रा.) में बढ़ती देखी गई। इस अध्ययन के परिणाम दर्शाते हैं कि बायोइथेनॉल के उत्पादन हेतु पार्श्विनय प्रजाति के जैविक पदार्थ का सफलता पूर्वक उपयोग हो सकता है।
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