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Pongamia pinnata seed residue – A low cost inedible resource for on-site/in-house lignocellulases and sustainable ethanol production

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ABSTRACT

Lignocelluosic biomass represents a promising feed stock for biofuels production. However currently the costs associated with lignocellulases represent a key limiting factor in the development of biomass conversion process. The aim of this work was to exploit *Pongamia pinnata* seed residue, a cheap inedible bioresource, for both lignocellulases and ethanol production. *Spingomonas echinoides* and *Iprex lacteus* were selected as novel sources of lignocellulases during solid state fermentation. Both organisms produced an array of lignocellulases (exoglucanase, endoglucanase, xylanase and laccase). Subsequent hydrolysis of the seed residue for sugars production using this crude enzyme from *S. echinoides* and *I. lacteus* were compared with those produced using commercial cellulase from *Aspergillus niger* (10 U g^{-1}), resulted in sugars yields of 233, 302 and 330 mg g^{-1}, respectively. Ethanol yields of 81.5, 104.5 and 157.6 mg g^{-1} and ethanol concentrations of 4.0, 5.3 and 7.9 mg mL^{-1} were achieved from the fermentation of the three hydrolyzates using *Saccharomyces cerevisiae*. The study demonstrated the feasibility of using the seed residue for enzyme preparation and its subsequent application in hydrolysis of the same seed residue and the potential of using the hydrolysis product for ethanol production.

1. Introduction

Depleting reserves of natural resources and the deleterious effects of fossil fuel burning on the environment have led to great interest in developing renewable fuels. Biomass represents a promising renewable material for biofuels, electricity, heat, chemicals and biochar production [28]. Consequently research has focused on aspects of biomass conversion that currently act as bottle necks to commercial development including: (i) optimization of the pretreatment process for efficient conversion of lignocellulosic biomass processing into fermentable sugars, (ii) development of stable and high specific activity enzyme production for hydrolysis, and (iii) development of large scale reactor systems at high solids loading [13]. Presently, biofuels from waste biomass, wood chips, forestry and agricultural residues via a biochemical route is the leading process strategy in many countries [3]. Significant improvements in fermentable sugars yield and cost reduction can therefore be expected, thus making large-scale processing of lignocellulosic substrates possible. The selection of conversion technologies and feedstock development are the most important factors in the commercialization of next-generation biofuels.

Currently, non-edible bioresources [18] including non-edible oil seeds are emerging as a potential feed-stock for biofuel industry, as both oil and seed residues can be exploited to produce biofuels. Non-edible resources for biofuels production has the advantage of not interfering with food supply and food pricing, nor impact on land use for feedstock supply to achieve sustainable biofuels production.

Pongamia pinnata, a non-edible, drought resistant tree is capable of adaptation to different climatic conditions. It belongs to legume family and is distributed in Asia, Australia and Pacific islands. *P. pinnata* seed oil represents a precursor for biodiesel production and has been widely studied as a potential renewable feedstock [2,6]. The residual seed is suitable for bioethanol production owing to its holocellulose composition [22]. Globally, many countries including Australia (Rural Industries Research and Development Corporation, Australian Government), India (National Oil Seeds and Vegetable Oils Development Board, the Ministry of Agriculture, India) and Hawaii (Source: Biodiesel crop implementation in Hawaii, Hawaii Agriculture Research Centre, 2006) have initiated techno-economic modelling and practices for *P. pinnata* plantation programs as a source of renewable feedstock for the biodiesel representing a sustainable energy supply. As a result the annual yield of the seeds reached 2,00,000 metric tons from India alone [7]. The seeds are composed of 30–35% oil which can be processed to biodiesel; the residue is of current interest in terms of hydrolysis to fermentable sugars for further biofuel production. Therefore, in the present work *P. pinnata* de-oiled seed residue feedstock was used for biofuel production through enzymatic hydrolysis for fermentable sugars, as a value addition of the seed residue to improve the economics of *P. pinnata* biodiesel production.

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Traditional biofuel production processes using lignocellulosic biomass involve pretreatment of biomass, hydrolysis of pretreated biomass, fermentation and the recovery of biofuel. All these operations impact on the final biofuel yield and economics of the process. Large amounts of water utilization and high energy requirement in heating the process material to the desired temperature in pretreatment process are major limitations of the current pre-treatment technologies [1]. Based on research reports on cellulosic bioethanol production, pretreatment is recognized as a major contributor to the total production cost of bioethanol [29]. The other major limiting factor in biomass to bioethanol process is the cost of cellulase enzymes for use in the hydrolysis of pretreated biomass [17]. To achieve cost effective biomass conversion for bioethanol production on-site/in-house enzyme production for the continuous supply of cellulases appears as one of the most cost effective options. A great deal of published material exists regarding cellulase production using different substrates by submerged or solid state fermentation using cellulolytic microorganisms (especially *Tricoderma reesei, Aspergillus niger* [27] and *Gracibacillus* species [30]) for application in lignocellulosic bioethanol production. Surprisingly, to the author's best knowledge there is no reported work on the production of lignocellulases using *P. pinnata* seed residue as carbon substrate nor the subsequent assessment of the hydrolysis of the seed residue using the enzymes prepared using *P. pinnata* substrate.

The aim of this study was to investigate the ability of two selected microorganisms, *Spingomonas echinoides*, a bacterial strain, and *Iprex lacteus* (ATCC® 11245™) a white rot fungi, to produce crude lignocellulase enzymes during growth on *P. pinnata* seed residue biomass. In addition the crude enzymes were employed in hydrolysis studies using the same biomass. Currently little information regarding lignocellulase production from these two micro-organisms exist. To assess the potential of these microorganisms as hydrolysis agents the amount of sugars formed (mg g^{-1} *P. pinnata* seed residue) by the action of crude enzymes from both *Spingomonas echinoides* and *Iprex lacteus* were compared with that from hydrolysis using commercial cellulase from *Aspergillus niger*. The products from the hydrolysis reaction were further processed in anaerobic fermentations using the yeast *S. cerevisiae* to assess the ethanol production yields from the seed residue.

2. Materials and methods

2.1. Materials

P. pinnata seed residue was obtained from a local producer in India and was used in the isolation of cellulolytic microorganisms, enzyme production and hydrolysis experiments. All chemicals, media, and commercial cellulase enzyme (from *Aspergillus niger*) used in the present study were of analytical grade and were purchased from Sigma Aldrich Pty. Ltd (NSW, Australia). *Spingomonas echinoides*, a Gram negative soil bacterium isolated from the seed residue, and *Iprex lacteus*, a white rot fungus obtained from RMIT Culture Collection, were used in the enzyme production experiments. Commercially available dry yeast from *S. cerevisiae* (Sigma Aldrich, NSW, Australia), was used in the fermentation experiments.

2.2. Crude enzyme production

Spingomonas echinoides and *Iprex lacteus* were used in the enzyme production experiments. *S. echinoides* and *I. lacteus* were selected based on pre-screening study including estimation of enzyme activities exhibited by the two strains in BH-cellulose liquid media (data not shown). Enzyme production was carried out in solid state fermentation mode using Bushnell-Hass salts medium (BH)-cellulose and BH-*P. pinnata* seed residue media at 30 °C. For inoculum preparation *S. echinoides* was grown in nutrient broth at 28 °C overnight and the pellet was obtained by centrifugation at 4000 rpm for 5 min at 4 °C. Media was inoculated with 3 mL *S. echinoides* suspension (nutrient free) of $OD_{600\ nm}$ 0.9. Fungal inoculum was prepared by growing fungi in potato dextrose broth for 3 days at 28 °C. Fungal biomass was harvested by centrifugation at 4000 rpm for 4 min and washed with sterile water three times to remove nutrients. Homogeneously suspended nutrient free fungal biomass (5 mL) in sterile water was used to inoculate the media for enzyme production; this corresponded to 0.3 g/L on dry basis.

In solid state fermentation experiments both cellulose and *P. pinnata* seed residue (10 g) were used as substrate and solid support and were moistened with BH liquid media (60 mL, pH 5.0) in 250 mL reagent bottles. Fermentations were carried out under static conditions at 30 °C for a period of 8 days. Cell free culture supernatants, prepared by centrifugation followed by filtration using a 0.45 μm filter, were analysed for exoglucanase, endoglucanase, β-glucosidase, xylanase, and laccase enzyme activities.

2.3. Enzyme activity measurement

Secreted extracellular enzyme was examined for different lignocellulase activities based on standard assay methods. Details of the methodology of the assays are presented in Table 1. Activities of different enzymes are defined as:

Table 1
Reagents and assay conditions in crude enzyme activity estimation.

Conditions	Assay				
	Exoglucanase	Endoglucanase	β-glucosidase	Xylanase	Laccase
Crude enzyme	0.5 mL	0.5 mL	0.5 mL	0.5 mL	0.5 mL
Substrate	0.5 mL 2% cellulose in sodium citrate buffer (0.05 M; pH 4.8)	0.5 mL 2% carboxymethyl cellulose in sodium citrate buffer (0.05 M; pH 4.8)	0.5 mL *p*-nitrophenyl-β-D-glucopyranoside (pNPG) (10 nM) in sodium citrate buffer (0.05 M; pH 4.8)	1% xylan in sodium citrate buffer (0.05 M; pH 4.8)	0.5 mL of guaiacol (0.02 M) in phosphate buffer (0.01 M; pH 6.5)
Temperature, °C	50	50	50	50	55
Time, min	60	60	15	60	10
Stopping reagent	DNS reagent (3 mL)	DNS reagent (3 mL)	Na_2CO_3 0.2M (2 mL)	DNS reagent (3 mL)	**No stopping reagent
Absorbance measurement	540 nm	540 nm	405 nm	640 nm	465 nm
Reference	[9]	[9]	[14]	[8]	[25]

$$Exo-\&Endo-glucanase\ activity,\ \ U/mL\ min = \frac{\mu mole\ glucose\ formed\ (U)}{mL\ enzyme.\ min}$$

$$\beta-glucosidase\ activity,\ \ U/mL\ min = \frac{\mu mole\ p-nitrophenol\ formed\ (U)}{mL\ enzyme.\ min}$$

$$Xylanase\ activity,\ \ U/mL\ min = \frac{\mu mole\ xylose\ formed\ (U)}{mL\ enzyme.\ min}$$

$$Laccase\ activity,\ \ U/mL\ min = \frac{Absorbance\ increase\ by\ 0.001\ (U)}{mL\ enzyme.\ min}$$

2.4. Enzymatic hydrolysis of P. pinnata seed residue

Hydrolysis experiments using *P. pinnata* seed residue were carried out in triplicate at 50 °C, the optimum temperature for cellulase activity, with mixing at 150 rpm. *P. pinnata* seed residue (5%, w/v) in sodium citrate buffer (pH 4.8) was pretreated at 121 °C for 15 min. Crude cellulases obtained from solid state fermentations using *S. echinoides*, *I. lacteus* and commercial cellulase from *A. niger* were employed in the hydrolysis of the pretreated seed residue. Enzymes at two different concentrations, 5 U and 10 U per g of *P. pinnata* seed residue (U g^{-1}), based on cellulase activity were used to study the effect of enzyme concentration on hydrolysis of the seed residue. Controls, without enzyme addition were routinely included. Samples were collected during the course of reaction and analysed for reducible sugars by the dinitrosalicylic acid (DNS) method [20].

2.5. Fermentation

Fermentation experiments were carried out for ethanol production by anaerobic fermentation of sugars released during the enzymatic hydrolysis of *P. pinnata* seed residue. Prior to fermentation, the pH of the hydrolysis product (liquid) was adjusted to 4.8 [4] by addition of the required amount of NaOH solution. The hydrolyzate, pH adjusted to 4.8, was subjected to fermentation using *S. cerevisiae* (2% w/v) at 35 °C. During the fermentation samples were collected every 24 h to monitor [20] conversion of sugars and to quantify ethanol formed using an ethanol assay kit (Sigma) [24].

2.6. Statistical analysis

In the present work, all the experiments were performed in triplicate. Statistical analysis of the experimental data was performed for mean, standard deviation and standard error and was used where required.

3. Results and discussion

3.1. Enzyme production in solid state fermentation

Solid state fermentation for enzyme production was carried out employing *S. echinoides* and *I. lacteus* on both cellulose and *P. pinnata* seed residue substrates separately. The fermentations were carried out for a period of 8 days at 30 °C, pH 5 and the enzyme activities detected at the end of the 8 day incubation are presented in Fig. 1. In Fig. 1 PIL, PSE, CIL and CSE represent solid state fermentations using *P. pinnata*-*I. lacteus*, *P. pinnata*-*S. echinoides*, cellulose-*I. lacteus* and cellulose-*S. echinoides,* respectively.

Crude enzyme preparation had appreciable levels of exoglucanase, endoglucanase, and xylanase activities in *P. pinnata* seed residue media inoculated with *S. echinoides* (PSE) and *I. lacteus* (PIL) but with very low laccase activity. With pure cellulose media (CSE and CIL in Fig. 1) the enzyme activities were significantly lower compared to that with *P. pinnata* seed residue media. Of the two organisms assessed, *I. lacteus* produced higher enzyme activities than *S. echinoides*. *S. echinoides* produced higher exoglucanase activity compared to endoglucanase while *I. lacteus* exhibited more endoglucanase than exoglucanase activity (Fig. 1). The highest exoglucanase, endoglucanase and xylanase activities obtained from *S. echinoides* and *I. lacteus* were 3.9, 2.7, 0.8 U mL^{-1} min^{-1} and 5.2, 8.2, 2.7 U mL^{-1} min^{-1}, respectively, in the presence of *P. pinnata* seed residue media (Fig. 1).

Enzyme production by solid state fermentation is a well-adapted process and has significant advantages over submerged fermentation [23]. Solid state fermentation processes are unaffected by the purity/impurity level of the substrate resulting in high concentrated enzyme production. The present investigation revealed similar results, with the production of concentrated (exhibiting high activity) lignocellulases from solid state fermentation from both the strains. These activities were nearly 10–50 times greater than those observed in liquid cultures (data shown in Appendix A).

3.2. Enzymatic hydrolysis of P. pinnata seed residue

Hydrolysis reactions were carried out using crude enzyme from *S. echinoides* and *I. lacteus* from solid state fermentation and for comparison a commercial cellulase preparation (from *A. niger*) at two different loadings, 5 U, and 10 U per g of *P. pinnata* seed residue at 50 °C. Pretreatment of the seed residue prior to enzymatic hydrolysis resulted in to the formation of ~50 mg glucose per g of *P. pinnata* seed residue. Pretreatment enhances the digestibility of lignocellulosic biomass by improving access of the enzymes to the substrate [11]. Enhanced hydrolysis rates can be expected with a pretreatment that facilitates the breakdown of the rigid structure of lignocellulosic biomass allowing better access for cellulases. Hydrolysis of *P. pin-*

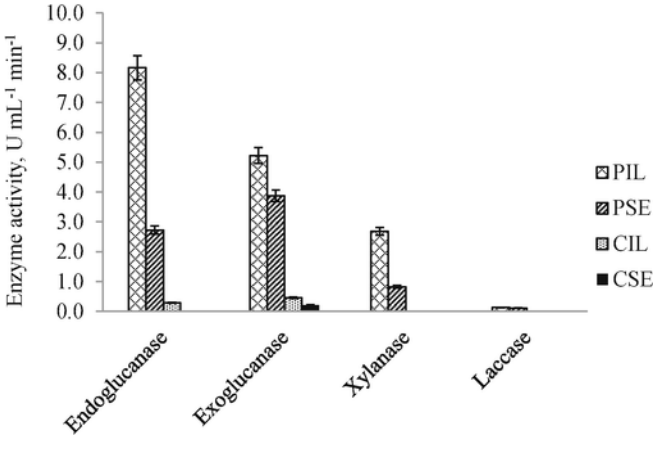

Fig. 1. Crude enzyme activities from solid state fermentations using *P. pinnata*-*I. lacteus* (PIL), *P. pinnata*-*S. echinoides* (PSE), cellulose-*I. lacteus* (CIL), cellulose-*S. echinoides* (CSE).

nata seed residue in terms of the amount of reducing sugars formed per g of seed residue i.e. sugars yield (mg/g) in the presence of different enzymes at two different enzyme concentrations is presented in Fig. 2.

In the presence of crude enzyme from *S. echinoides* sugar yields from *P. pinnata* seed residue reached 171 mg g^{-1} and 233 mg g^{-1}, at enzyme concentration of 5 and 10 U g^{-1} respectively. Crude enzyme from *I. lacteus* resulted in higher values, 280 mg g^{-1} and 306 mg g^{-1} sugar formation at 5, 10 U g^{-1} enzyme concentration, respectively. These values were comparable to those obtained with the commercial cellulase preparation, with a sugar yield of 331 mg g^{-1} at 10 U g^{-1} and 302 mg g^{-1} sugars yield with the enzyme at 5 U g^{-1}.

The results suggest that the presence of exoglucanase, endoglucanase and xylanase in the crude enzyme samples can effectively breakdown the cellulose and hemicellulose constituents present in *P. pinnata* seed residue for application in bioethanol production. In our earlier work [22], sulphuric acid hydrolysis of the same seed residue resulted sugars yield of 245 mg g^{-1} whereas in the present work enzymatic hydrolysis showed a yield of ~300–330 mg/g. Improved sugars yield were achieved from enzymatic hydrolysis compared to chemical hydrolysis of the same seed residue. The results were further compared with the sugars yield (343 mg sugars/g rice straw) from rice straw in a two stage dilute sulphuric acid hydrolysis under high pressure (30 bar) [15]. Although chemical hydrolysis of lignocellulosic biomass has been reported with good sugar yields [15,22], but this approach has some limitations, including, (i) formation of sugar degradation products which show a negative effect on microbial activity during fermentation; (ii) the acid used in the process is not recycled; (iii) neutralization of the acid results the formation of salts. All these factors together motivated the search for an environmentally and economically feasible process strategy to address all or some of these disadvantages. Among the available biomass conversion processes enzymatic hydrolysis is gaining attention because of the highly specific nature of enzymes and mild temperatures (50 °C) of the process. However, the cost of the enzymes is a major disadvantage of the enzymatic conversion of biomass. Therefore, the present work was aimed at on site/in house preparation of cellulase enzymes using *P. pinnata* seed residue as carbon source, together with its characterization and further application of the prepared enzyme mix for hydrolysis of the same seed residue. Therefore, enzymatic hydrolysis using crude enzyme preparation is a promising option for conversion of *P. pinnata* seed residue to sugars and the seed residue has the potential to compete with the lignocellulosic feed-stocks for ethanol production.

3.3. Fermentation

The three liquid products from the enzymatic hydrolysis of *P. pinnata* seed residue using crude enzyme prepared from *S. echinoides*, *I. lacteus* and cellulase from *A. niger* were fermented using *S. cerevisiae* for 4 days under anaerobic conditions. The conversions of sugars and ethanol formed during the course of fermentations are presented in Fig. 3 (a) and (b), respectively. The stoichiometry of glucose conversion to ethanol and carbon dioxide during fermentation production is presented below. According to the stoichiometry 1 mol of glucose results the formation of 2 mol of ethanol i.e. 180 g of glucose may lead to 92 g of ethanol.

$$C_6H_{12}O_6 \xrightarrow{S.\ cerevisiae} 2C_2H_5OH + 2CO_2$$

In Fig. 3 SE, IL and AN represent fermentation of the liquid product from enzymatic hydrolysis of *P. pinnata* seed residue employing crude enzyme from *S. echinoides*, *I. lacteus* and cellulase-*A. niger*, respectively. During the course of the fermentation of the enzymatic hydrolysis product, the liquid samples were analysed for both glucose utilization and ethanol formation. Generally, the fermentation of biomass hydrolysis products using *S. cerevisiae* results in the formation of secondary fermentation by-products such as, acetaldehyde, glycerol, acetic acid, formic acid, lactic acid and other alcoholic products [19].

A rapid conversion of sugars, ~80%, was observed in AN fermentation within 1 day followed by a slow increase in the conversion. During AN fermentation the conversion plateaued out after 2 days and a maximum conversion of 93% was observed at the end of day 4. Fermentation of SE and IL resulted into a sugars conversion of ~50% after 1 day and a slow increase in the conversion was observed thereafter. An almost similar sugar conversion profile was observed dur-

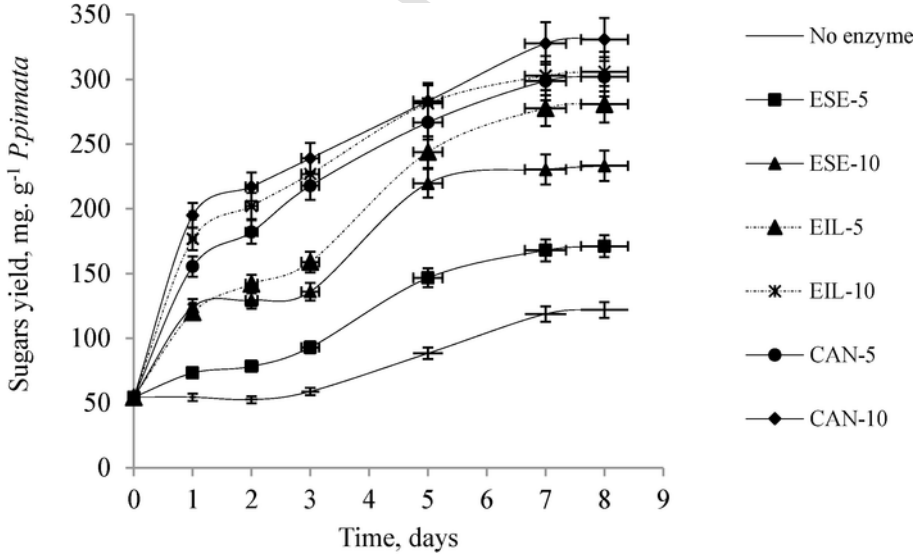

Fig. 2. Sugar yield (mg g^{-1}) from hydrolysis of *P. pinnata* seed residue using crude enzyme from *S. echinoides* (ESE), *I. lacteus* (EIL), and commercial cellulase from *A. niger* (CAN) at 5 and 10 U g^{-1} concentration.

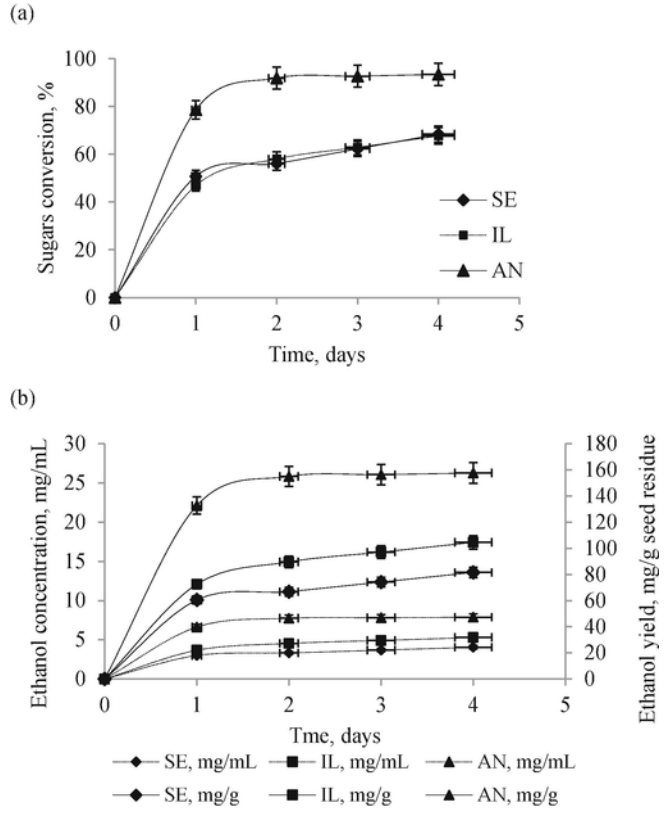

Fig. 3. (a) Sugars conversion; (b) Ethanol concentration and yield during fermentation using *Saccharomyces cerevisiae*.

ing the fermentation of SE and IL and a maximum conversion of ~68% was observed at the end of day 4. Ethanol yield in terms of mg ethanol formed from g of the seed residue is presented in Fig. 3b. Highest ethanol yield was observed from AN fermentation, 157.6 mg/g seed residue. In the case of fermentation of hydrolyzates from crude enzyme treatment ethanol yields of 81.5 and 104.5 mg/g were observed from SE and IL, respectively. Final concentration of ethanol of 4.0, 5.3 and 7.9 mg/mL were observed from SE, IL and AN fermentation, respectively, Fig. 3b.

The highest sugar conversion and ethanol yield from fermentation of AN is attributed to the presence of hexose sugars alone in AN. It is well reported that cellulases are highly specific and are able to hydrolyse cellulose alone resulting in the release of hexose sugars from the cellulose matrix. Cellulose is a polymer of hexose subunits. When cellulase from *A. niger* was employed in hydrolysis of *P. pinnata* seed residue the enzyme will ultimately act on the cellulose portion of the seed residue and resulted into the formation of hexoses which can easily be fermented by *S. cerevisiae*. In contrast in the case of SE and IL, the presence of hexoses and pentoses can be expected in the products as crude enzyme preparation from *S. echinoides* and *I. lacteus* as both produce cellulase and xylanase activities which can act on both the cellulose and hemicellulose portion of the seed residue resulting in the formation of hexose and pentose sugars. *S. cerevisiae* is a highly efficient in hexose sugars fermentation, but it is unable to ferment pentose sugars. This likely explains the lower sugar conversion and ethanol yield from the fermentation of SE and IL [26]. These results suggest that the onsite enzyme production strategy will also require a co-fermentation technology for ethanol production from both pentose and hexose sugars to improve ethanol yields. The co-fermentation of hexose and pentose sugars can be possible by applying engineered/recombinant yeast strains in bioethanol production from lignocelluloses, an area of active research at the present [10].

Techno-economic analysis of lignocellulosic bioethanol production cost report says that the enzymes cost about $ 132 per cubic meter of ethanol when the enzymes supplied by commercial enzyme manufacturers, Novozymes [5]. In contrast, in the case of on-site enzyme production the overall cost of enzyme was reported to be $ 90 per cubic meter of ethanol that is lower than Novozymes. Humbird et al. [12] had estimated the cost of enzyme prepared from pure glucose as the carbon and energy source and reported that the carbon source is the most significant expense in enzyme production. In the present work, *P. pinnata* seed residue, after extracting oil from the seeds, was utilised as the carbon source during the enzyme preparation. *P. pinnata* seeds costs around \$0.06 kg^{-1} and the selling price of oil extracted from the seeds is \$0.06 L^{-1} (\$0.065 kg^{-1}) [21]. De-oiled *P. pinnata* seed residue can be exploited in place of commercially available substrate for enzyme production to improve the overall economics of the seed residue-based process. Therefore, the work suggests that on-site/in-house enzyme preparation using *P. pinnata* seed residue represents a promising option in enzymatic conversion of the same seed residue for further application in bioethanol production.

Overall, *P. pinnata* seed residue has significant potential to be exploited as a feedstock for the production of sugars. The on-site/in house enzyme production using *S. echinoides* and *I. lacteus* also represents a promising strategy to make the enzymatic hydrolysis process economically feasible. Importantly, the process adopted in the present work is environmentally friendly as no chemicals or organic solvents were employed in pretreatment and enzymatic hydrolysis of the seed residue. However the fermentation of the total sugars formed during hydrolysis using crude enzyme preparations needs improvement to achieve better ethanol yield, which can be possible using engineered *S. cerevisiae* strains.

4. Conclusions

High cost of cellulases, a major limiting factor, in the seed residue conversion process can be addressed by on-site/in-house enzyme preparation. *Spingomonas echinoides* and *Iprex lacteus* had the potential of producing highly active lignocellulases for application in the hydrolysis of the seed residue. Crude enzyme from *S. echinoides, I. lacteus,* and commercially available cellulase from *A. niger* resulted in appreciable sugars yield at a minimum enzyme concentration (10 U g^{-1}) and ethanol yields of 82, 104 and 158 mg g^{-1} seed residue were observed, respectively. *P. pinnata* seed residue is a potential substrate for both lignocellulases and sugars production for further application in bioethanol preparation.

Appendix A.

	Endoglucanase	Exoglucanase	Xylanase	Laccase
Enzyme activities in submerged fermentation, U mL^{-1} min^{-1}				
SBF	0.129	0.097	0.058	0.004
SBB	0.000	0.057	0.000	0.097
SCF	0.180	0.165	0.000	0.000
SCB	0.000	0.051	0.000	0.000
Enzyme activities in solid state fermentation, U mL^{-1} min^{-1}				
PIL	8.166	5.223	2.681	0.129
PSE	2.729	3.871	0.825	0.116
CIL	0.289	0.457	0.000	0.000
CSE	0.000	0.216	0.000	0.000

SBF: Submerged fermentation using *P. pinnata*-*I. lacteus*.
SBB: Submerged fermentation using *P. pinnata*-*S. echinoides*.
SCF: Submerged fermentation using cellulose-*I. lacteus*.
SCB: Submerged fermentation using cellulose-*S. echinoides*.

PIL: Solid state fermentation using *P. pinnata-I. lacteus*.
PSE: Solid state fermentation using *P. pinnata-S. echinoides*.
CIL: Solid state fermentation using cellulose-*I. lacteus*.
CSE: Solid state fermentation using cellulose-*S. echinoides*.

Uncited reference

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