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Value addition to *Pongamia* biodiesel industry through bioethanol production from pressed oil cake using *Bacillus cereus*

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Abstract

Pongamia pinnata, a tree bearing oil seeds, has been one of the prime focus among nonedible oil sources for biodiesel production in most parts of India. The oil cake obtained after the extraction of oil serves as an adequate source of cellulose, hemicellulose, proteins, lipids, and starch. The scarce literature data available suggest very low conversion rate of complex sugars to fermentable sugars and further to bioethanol. In the present work, a sulfuric acid hydrolysate obtained from *Pongamia* oil cake (POC) under microwave irradiation was anaerobically fermented using *Bacillus cereus* ATCC 14579. Under anaerobic and acidic conditions, the process parameters affecting the bioethanol production efficiency (BPE) were screened and optimized using statistical experimental designs. Of the seven parameters screened (glucose concentration, yeast extract as nitrogen source, agitation speed, pH, inoculum size, fermentation time, and temperature), only 3 were found to be significant and to increase BPE. The significant parameters were optimized using the Box-Behnken experimental design to obtain an optimal condition of 9 g/l reducing sugars, pH 4.6 maintained with acetate buffer, and fermentation period of 51 h that yielded 92% BPE with 4.2 g/l ethanol. Moreover, it was observed that the use of a buffer system to maintain broth pH, especially acetate buffer, significantly improved BPE. Low concentration of fermentation inhibitors is the key factor for improved BPE. The research outcome suggests that the production of dual fuel from *Pongamia pinnata*, viz. biodiesel and bioethanol, could not only improve the process economics but also reduce waste generation.

Key words: *Pongamia pinnata*, oil cake hydrolysate, bioethanol, *Bacillus cereus*, value addition

Introduction

Bioethanol is one of the second-generation biofuels produced to a large extent through microbial fermentation of treated lignocellulosic biomass. Technologies for the conversion of cellulose to bioethanol have reached commercial production stage, with the production of several million gallons per year for end user consumption (Brown and Brown, 2013). However, contemporary technologies to explore novel feedstocks, their processing technologies, followed by fermentation schemes and scale-up continue to increase (Schell et al., 2016). Several under-utilized feedstocks such as chili post-harvest residues, citrus fruit waste, hazelnut husks, fresh-water biomass like hyacinths, seaweeds, rice straw, banana waste, and sweet sorghum have been reported to be used in bioethanol production (Sindhu et al., 2018; John et al., 2019; Passoth and Sandgren, 2019; Sunwoo et al., 2019). However, high production (pretreatment) costs adversely affect the commercialization of second generation (2G) bioethanol, similar to the availability of raw material throughout the year, quantity, and cost of feed biomass. For example, Brazil, with its experience of more than 40 years in bioethanol production at the commercial scale, depends on sugarcane molasses (Lopes et al., 2016). In Asian and African countries, rice straw (Singh et al., 2017) and rice husks (Omidvar et al., 2016) are the locally available feedstocks for bioethanol production. The waste generated from oil palm tree is the

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principal source for bioethanol production in countries such as Indonesia, Thailand, and Malaysia (Shahirah et al., 2015). Similarly, in India, sugarcane molasses has been the major source of commercial ethanol production. However, the revised Indian "National Biofuel Policy– 2018" (MNRE, 2018), endorses the use of waste food grains, waste potato, surplus sugarcane, agricultural residues, and under-utilized nonedible sources for bioethanol production. The same policy also thrusts upon the usage of nonedible oil seeds such as *Jatropha* and *Pongamia pinnata* (Karanja) for biodiesel production. One of the major byproducts in biodiesel industry is the oil cake, which is rich in cellulose, starch, hemicellulose, proteins, and lignin (Mishra and Mohanty, 2018). In Indian biodiesel production sector, *Pongamia* is one of the major feedstocks because of its availability and annual yield of seeds (Iyyaswami et al., 2013).

P. pinnata (Karanja) belongs to Leguminosae or Fabaceae family, and it is an evergreen tree with an average height of 10 to 15 m. Tropical temperature conditions in India are highly suitable for its growth (Harreh et al., 2018). *Pongamia* starts producing fruits within 5–8 years after planting. The seeds are oval-shaped and clustered in eye-shaped pods, and they are usually 1.5 cm long (Dwivedi et al., 2011). The annual yield of seeds is estimated to be 8–90 kg per tree depending on the age of tree, and the overall productivity in India is of more than 0.2 MMT (in 2012). Extraction of brownish orange oil from these seeds by using expellers yields 0.3 kg oil per kg seeds, which is not suitable for human and animal consumption (Chandrashekar et al., 2012). While the oil is used for biodiesel production, the coproduct de-oiled cake is useful for 2G bioethanol production (Fig. 1). There are only a few instances where *Pongamia* oil cake (POC) is reported for bioethanol production, and all of them used the yeast *Saccharomyces cerevisiae*. Muktham et al. (2016) reported 41.3% yield of bioethanol from reducing sugars obtained through acid hydrolysis of POC. Hydrolysis of POC using crude array of lignocellulases enzyme obtained from *Iprex lacteus and Spingomonas echinoides* yielded 104.5 and 81.5 mg per g of biomass corresponding to ethanol titer of 5.3 and 4.0 g/l, respectively (Radhakumari et al., 2017). In another study, 10% ethanol yield at 32 °C was reported from POC hydrolysate obtained by sulfuric acid (0.5%) treatment using the steam cooking method (Doshi and Srivastava, 2013). Importantly, the ethanol yield obtained using yeast did not improve beyond 45%.

Adaptive evolution of microorganisms by increasing their tolerance to several stresses in the fermentation medium has been used as an approach to produce ethanol (Moreno et al., 2019; Sunwoo et al., 2019). Not only the bioethanol yield, but tolerance to ethanol is also one of the prime factors in process improvement. Ire et al. (2016) isolated native strains of *Bacillus cereus* and *Bacillus thuringiensis* exhibiting cellulolytic and xylanolytic activities from agricultural waste-fertilized soils and used the coculture to demonstrate the production of 15 g/l bioethanol from steam-exploded bagasse. Moreover, *B. cereus* strain showed ethanol tolerance up to 6% (v/v). By using phenotypic microarray experiments followed by microbial growth in minimal medium, Mols et al. (2010) showed that *B. cereus* ATCC 14579 strain could utilize several mono and oligosaccharides, asparagine, and glutamine. They also reported that *B. cereus* ATCC 14579 strain exhibited high tolerance to stress induced by acidulants, especially acetic acid stress that activated the ethanol production pathway. High tolerance of this microorganism to acidic conditions as well as good ethanol titer could be beneficial in producing bioethanol from lignocelluloses.

In the present study, *B. cereus* ATCC 14579, a well-known microorganism that causes food spoilage is reported to produce bioethanol from POC hydrolysate obtained through sulfuric acid treatment under microwave irradiation. Process conditions affecting bioethanol production were studied by orthogonal experiments and optimized by statistical modeling to improve the bioethanol production efficiency (BPE).

Materials and methods

Chemicals and microorganism

All the chemicals were of reagent grade and were obtained from Merck India, HiMedia, or Loba Chemie, and they were used without any further modification. In all fermentation experiments, the native strain of *B. cereus* ATCC 14579 was used as the fermenting microorganism. This strain was isolated from raw honey by co-researchers and characterized at MTCC & GeneBank, CSIR-IMTECH, Chandigarh, India, by using 16s RNA gene sequencing. The microorganism was subcultured on glucose-supplemented minimal nutrient medium (HiMedia, India) for 24 h before its use as a starter culture. Briefly, the final growth medium was composed of 5 g/l

glucose as carbon source, 5 g/l peptone, 5 g/l NaCl, 1.5 g/l HM peptone B (eq. beef extract), and 1.5 g/l yeast extract, and the pH was adjusted to 5.

To maintain anaerobic conditions in all the fermentation experiments, hot fermentation medium after sterilization was quickly overlaid with mineral oil sterilized overnight in a hot air oven at 80°C (Ahn and Burne, 2007).

Processing of Pongamia pressed oil cake (POC)

Pongamia oil cake, obtained from Bioenergy Research and Demonstration Centre, Nitte, Udupi, India, was oven-dried, powdered using a blender, and sieved to a size of 0.7 to 1.0 mm particle size. By using an optimized microwave-assisted sulfuric acid treatment technique, POC hydrolysate was prepared for all fermentation experiments (Kamath et al., 2018a). In brief, an Erlenmeyer flask with 75 ml mixture containing 6% (w/v) powdered POC (mean particle size 850 μm) and 2.75% (v/v) sulfuric acid was irradiated in a microwave oven for 5.5 min at 280 W microwave power. The POC hydrolysate was analyzed for reducing sugar, glucose and xylose content, sugar degradation products (hydroxymethylfurfural (HMF) and furfural), and total protein concentration. The pH of the hydrolysate was adjusted to 5.0 ± 0.2 by using 5N NaOH solution, and the hydrolysate was then used as a medium for ethanolic fermentation.

Fermentation experiments

In a typical fermentation experiment, screw-capped culture bottles were filled with 100 ml of POC hydrolysate, and yeast extract was added to a final concentration of 3 g/l. After sterilization at 15 psig pressure for 20 min, 4 ml mineral oil was added on top of the hot medium to maintain anaerobic condition. To maintain minimum dissolved oxygen, mineral oil was added soon after the sterilization to the culture flasks. Absence of dissolved oxygen was monitored using a few drops of resazurin solution. To vary the glucose concentration (from 3 to 9 g/l) in the fermentation medium, the POC hydrolysate was appropriately diluted. Two milliliters of the bacterial starter culture (quantified in terms of OD_{610}) was added to the POC hydrolysate layer by using a sterile pipette, and the hydrolysate was agitated at 100 rpm for 24 h. The incubation temperature was maintained at 30°C. The fermentation product stream was quantitatively analyzed for reducing sugar and ethanol concentrations. Bioethanol yield and BPE were calculated using Eq. 1 and Eq. 2, respectively. Theoretical yield (mass basis) was considered as 0.511, based on the conversion of 1 mole (or 180 g) of glucose to 2 moles (or 92 g) of ethanol. BPE was used as a response variable to screen the variables and to optimize the significant factors. The same procedure was used for all the subsequent experiments.

$$\text{Bioethanol yield [gE/gG]} = = \frac{\text{ethanol concentration in broth [g/l]}}{\text{fed glucose concentration [g/l]}} \quad (1)$$

$$\text{Bioethanol production efficiency [\%]} = = \frac{\text{bioethanol yield}}{\text{theoretical yield}} \times 100 \quad (2)$$

Factorial screening experiments

Potential variables that could affect ethanol production from POC under anaerobic condition were identified as reducing sugar concentration in the hydrolysate, yeast extract (nitrogen source) content, inoculum size (OD_{610}), broth pH, operating temperature, and incubation time. The Plackett-Burman experimental design consisting of 12 experimental runs and 2 center points was used to screen the parameters that affect ethanol production from POC hydrolysate. The experimental variables and their levels used in this experimental design are listed in Table 1. The orthogonal experiments were designed and analyzed at 5% significance level by using the software Minitab v15.0. The randomized experiments were conducted in duplicate, and the mean response was used to identify the main effects through analysis of variance.

Optimization experiments

The screening experiments identified three factors, namely glucose concentration, broth pH, and incubation time, as significant parameters that affect bioethanol production. Hence, the Box-Behnken experimental design with 3-factor and 3-level was used to optimize the significant parameters. The orthogonal experiments comprising 12 experimental runs and 3 repeated center points were used to optimize the process parameters with an objective to maximize BPE. The factors used for the optimization experiments and their levels are listed in Table 2. The three levels of design parameters were as follows: glucose concentration (3, 6, and 9 g/l), broth pH (4, 5, and 6), and incubation time (24, 48, and 72 h).

Table 1. Plackett-Burman screening design: factors and their levels used

Parameter	Unit	Symbol	Code and levels		
			low [−1]	mid [0]	high [+1]
Glucose concentration	g/l	A	3	6	9
Yeast extract	g/l	B	0.0	1.5	3.0
Agitation speed	rpm	C	50	100	150
Broth pH	–	D	4	5	6
Inoculum size	OD_{610}	E	0.25	0.50	0.75
Incubation time	h	F	24	48	72
Temperature	°C	G	25	30	35

Table 2. Box-Behnken optimization experiments: factors and their levels used

Parameter	Symbol	Code and levels		
		low [−1]	mid [0]	high [+1]
Glucose concentration [g/l]	X_1	3	6	9
Broth pH	X_2	4.0	5.0	6.0
Incubation time [h]	X_3	24	48	72

In contrast to screening experiments, broth pH was first adjusted using NaOH solution and then maintained to a equired pH (4, 5, or 6 according to the experimental design) by adding acetate buffer to 4 mM final concentration. The inoculum size corresponding to OD_{610} of 1.0 was used in all the experiments, which ensured constant inoculum size in all experiments. All samples were agitated at 100 rpm at room temperature 30 ± 2°C. The fermentation product was distilled, and ethanol concentration in the original broth was quantified. The randomized experiments were run in duplicate, and their mean value was fit to a full quadratic model (Eq. 3), where X_i and X_j are coded factor levels and β_0, β_i, and β_j are coefficients.

$$BPE \text{ or } Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i \quad (3)$$

Analytical methods

Samples were collected from the fermentation unit and centrifuged at 11 200 *g* for 15 min at 6°C. The supernatant was used for the quantitative estimation of sugars, toxic substances, proteins, and ethanol content. Samples were diluted before analysis according to the standard protocols to match the calibration range. Reducing sugar content was estimated using the DNS method (Miller, 1959), while protein content was estimated using Bradford's method (Bradford, 1976). The amount of hexoses and pentoses in the sample was simultaneously estimated using o-toluidine reagent as described by Goodwin as glucose and xylose equivalents (Goodwin, 1970). HMF content was estimated according to White's method (White, 1979), and furfural content was determined using the HCl-aniline method (Al-Showiman, 1998). Ethanol in the fermentation broth was first distilled and then quantitatively estimated by gas chromatography (GC). GC was performed by injecting 0.2 µl of sample into an HP-5 column maintained isothermally at 150°C. The injector and FID temperature were maintained at 250°C and the nitrogen carrier gas flow rate at 25 ml/min.

Statistical analysis

All data are presented as mean ± SD based on at least 2 or 3 repeated experiments. Statistical analysis of factorial experiments and process optimization were carried out using Minitab v15.0. A significance level of 5% was used for all statistical tests.

Results and discussion

Second-generation biofuels produced from nonedible oil seed sources are gaining importance. *P. pinnata*,

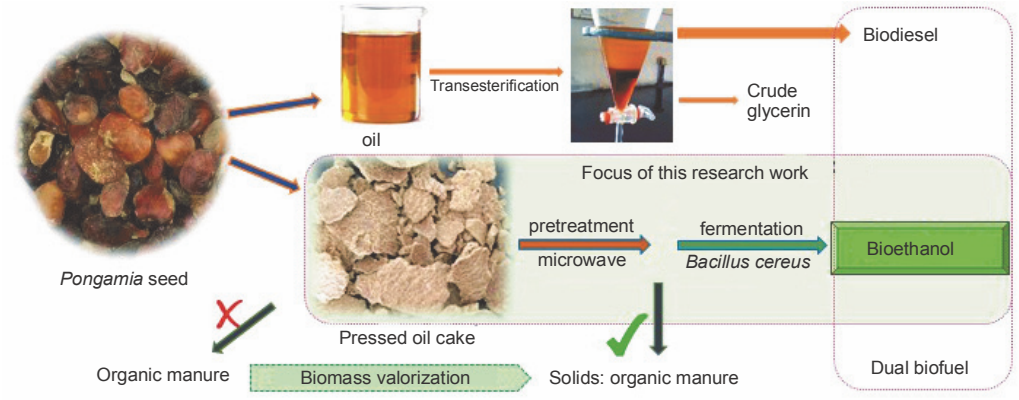

Fig. 1. Scheme of value addition to *Pongamia*-based biofuel production

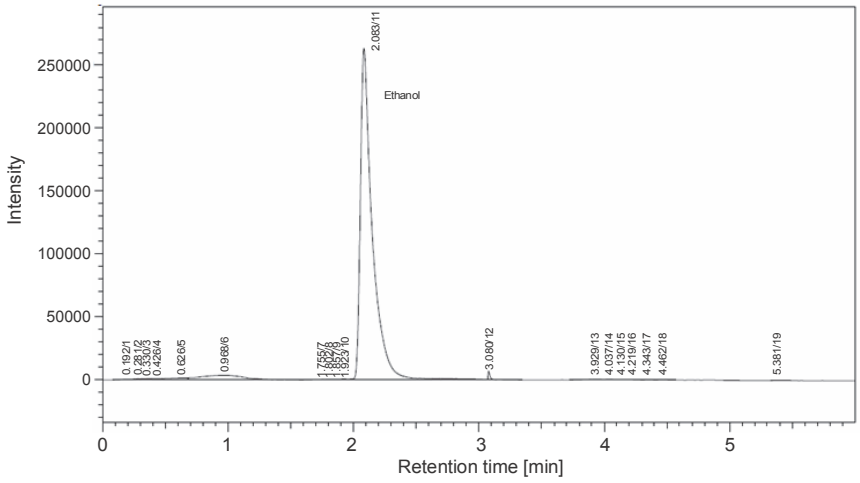

Fig. 2. Gas chromatogram of fermentation distillate showing an ethanol peak

a nonedible oil seed-borne tree, is being used for the mass production of oil seeds and biodiesel in India and other Asian countries. The oil cake leftovers are suitable for the production of bioethanol; however, the available literature data show that the bioethanol conversion efficiency is as low as 45% (Radhakumari et al., 2017; Kamath et al., 2018b). Here, we report the use of hydrolysate produced from microwave-assisted sulfuric acid digestion for bioethanol production and improvement in conversion efficiency up to 92%. The major reason for improved product yield is attributed to the presence of low amounts of fermentation inhibitors and the capability of *B. cereus*, a bacterium that has not yet been used for bioethanol production from cellulosic sources.

Ethanol production from POC hydrolysate using B. cereus

P. pinnata pressed oil cake or POC powder was acid digested under microwave irradiation to prepare POC hydrolysate required for all fermentation experiments as described in the experimental section. Analysis of the POC hydrolysate showed a composition of 9.41 ± 0.19 g/l reducing sugars with mean glucose and xylose equivalents of approximately 7.20 g/l and 2.70 g/l, respectively, indicating a lower amount of pentoses than that of hexoses. Moreover, the hydrolysate contained 1.95 g/l total soluble protein, 350 mg/l HMF, and 70 mg/l furfural. The addition of sulfuric acid during acid digestion decreased the solution pH to a range of 2 to 2.5. After adjusting the broth pH to 5, reducing sugar concentration to 9 g/l, and yeast extract to 3 g/l, anaerobic fermentation carried out for 72 h produced 1.65 g/l ethanol corresponding to 44.8% BPE. A sample gas chromatogram for fermentation sample is shown in Figure 2 with 2.08 min retention time for ethanol.

The proximate composition of *P. pinnata* pressed oil cake (POC) used in the present study is approximately

Table 3. Experimental combination used in PBD and the results of each experiment shown as mean ethanol concentration and % BPE to screen significant factors affecting bioethanol production

Standard order	Run order	Factorial variables (coded level) [a]							BPE [%]
		A	B	C	D	E	F	G	
1	3	1	−1	+1	−1	−1	−1	+1	49.3
3	14	−1	+1	+1	−1	+1	−1	−1	37.4
4	12	1	−1	+1	+1	−1	+1	−1	24.7
5	6	1	+1	−1	+1	+1	−1	+1	24.3
6	8	1	+1	+1	−1	+1	+1	−1	58.1
7	9	−1	+1	+1	+1	−1	+1	+1	17.5
8	4	−1	−1	+1	+1	+1	−1	+1	10.8
9	10	−1	−1	−1	+1	+1	+1	−1	14.0
10	7	1	−1	−1	−1	+1	+1	+1	55.4
11	2	−1	+1	−1	−1	−1	+1	+1	34.9
12	5	−1	−1	−1	−1	−1	−1	−1	34.1
13	1	0	0	0	0	0	0	0	38.9
14	13	0	0	0	0	0	0	0	40.5

[a] Factors: A– glucose concentration, B – yeast extract, C – agitation speed, D – broth pH, E – inoculum size, F – incubation time, G – temperature; levels used: high (+1), mid (0) and low (−1); response: BPE – Bioethanol Production Efficiency

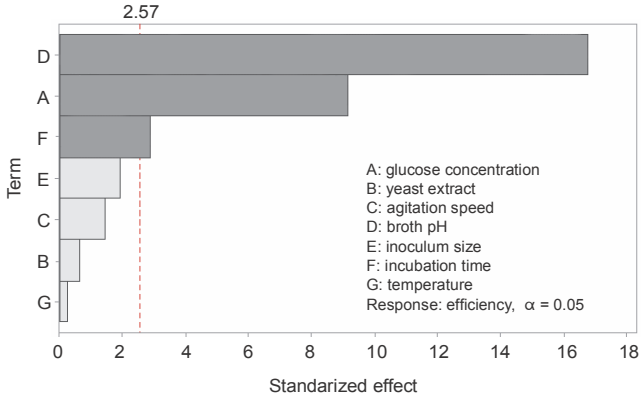

Fig. 3. Pareto chart showing significant and nonsignificant terms or process parameters obtained through screening experiment (PBD); bars with crossed wires represent significant terms and plain bars represent nonsignificant terms, critical $t_{(\alpha = 0.05,\ df = 5)} = 2.57$

60% complex carbohydrates and 4.2% lignin by weight (Kamath et al., 2018b). Ordinarily, the sugar degradation products such as HMF and furfural are produced during acid digestion and are considered to be toxic for ethanolic fermentation. In contrast, the low concentration of toxic substances in this case was due to the thermal effects caused by the microwave radiation during the hydrolysis process (Kamath et al., 2018a, 2018b).

Screening for significant factorial variables

B. cereus ATCC 14579 can grow on several types of sugar molecules under anaerobic conditions. The preliminary experiments with POC hydrolysate confirmed this observation, but with BPE as low as 44.8%. Apparently, the bioethanol yield could be increased by proper controlling of process variables or factors affecting fermentation. The Plackett-Burman design (PBD), a fractional factorial orthogonal design, with 7 factorial variables was used to screen the significant factors that influence BPE. Table 3 summarizes the experimental design conditions used during fermentation and the results (%BPE and ethanol concentration) obtained in each experiment. The lowest BPE (10.8%) corresponding to 0.17 g/l was obtained for an experimental combination of "Low (or −1)" settings for glucose concentration, yeast extract concentration, and incubation time and "High (or +1)" settings for agitation speed, broth pH, inoculum size, and temperature. The highest BPE (58.1%) corresponding to 2.67 g/l was obtained at "High (or +1)" settings for glucose concentration, yeast extract concentration, agitation speed, and inoculum size and "Low (or −1)" settings for broth pH and temperature. Similarly, for different combinations of factorial varia-

Table 4. ANOVA table to screen factorial variables using PBD

Source	Effect	DF	SS	MS	F	*P*
Model		8	2840.44	355.05	49.31	< 0.001 *
Linear		7	2734.59	390.66	54.26	< 0.001 *
glucose concentration	14.196	1	604.55	604.55	83.96	< 0.001 *
yeast extract	0.972	1	2.83	2.83	0.39	0.558
agitation speed	2.220	1	14.78	14.78	2.05	0.211
broth pH	−25.994	1	2027.12	2027.12	281.53	< 0.001 *
inoculum size	2.934	1	25.82	25.82	3.59	0.117
fermentation time	4.440	1	59.13	59.13	8.21	0.035 *
temperature	0.345	1	0.36	0.36	0.05	0.832
Curvature		1	105.85	105.85	14.70	0.012 *
Error		5	36.00	7.20		
lack-of-fit		4	34.75	8.69	6.93	0.277
pure error		1	1.00	1.25	1.25	
Total		13	13.00	2876.44		

* Significant at 5% significance level ($P < 0.05$)

bles, the ethanol concentration and the corresponding %BPE were recorded as shown in Table 3. To identify the statistically significant factorial variable, statistical analyses were carried out with %BPE as the response variable.

BPE was fit to a linear main effects model, and ANOVA was performed with 5% significance level. The coefficient of determination (96.8%) showed that the experimental data fitted well to the main effects model, with 3.2% nondeterministic error. Statistical tests and ANOVA used to screen the parameters are summarized in Table 4. A nonsignificant lack-of-fit test [$F_{4,1} = 6.93$, $P > 0.05$] indicated larger variances shown by the controlled factors than that by the natural variances. Three factors were significant at 5% significance level, viz. glucose concentration, broth pH, and fermentation time. In addition, a significant curvature term suggested the presence of an optimal point within the experimental range used. Figure 3 depicts a Pareto chart with the standardized effect of each factor and their relevance with respect to critical t value [$t_{(crit)df = 1, \alpha = 0.05} = 2.57$]. Here, broth pH, glucose concentration, and fermentation time showed a significant effect on bioethanol production. Because glucose is the precursor for biomass growth and bioethanol production, it tends to increase bioethanol production (Namadi, 2013; Ire et al., 2016); hence, a strong positive effect was noted. Ire et al. (2016) found that bagasse loading up to 4% w/v increased ethanol yield. Furthermore, *B. cereus* shows high resistance to higher concentration of glucose (Mols et al., 2007) and ethanol (Ire et al., 2016).

A significant increase in the production of ethanol by *B. cereus* was observed as pH decreased from 6 to 4, indicating the tendency of the bacterium to produce ethanol under acidic environment or acidic stress. *B. cereus* strain ATCC 14579 is known to exhibit strong resistance to acidic stress under anaerobic condition (Mols et al., 2007; Mols and Abee, 2011). Further, this microorganisms showed lesser cytotoxicity to acetic acid up to 15 mM than to HCl (2 mM) and lactic acid (2 mM) when grown in minimal medium (Mols et al., 2007). The use of acidic condition could be economically beneficial at higher scales, as it requires lesser quantity of neutralizing agents and prevents contamination by other bacteria that are less tolerant to strong acidic condition. A significant ($P = 0.035$) main effect (4.44) for incubation time indicated an increase in %BPE as the fermentation continued from 24 h to 72 h. It was expected that over the period of fermentation, reducing sugars would be completely converted to ethanol, thereby yielding high BPE. However, bioethanol yield (in terms of BPE) increased up to 58% at 72 h starting from the initial reducing sugar

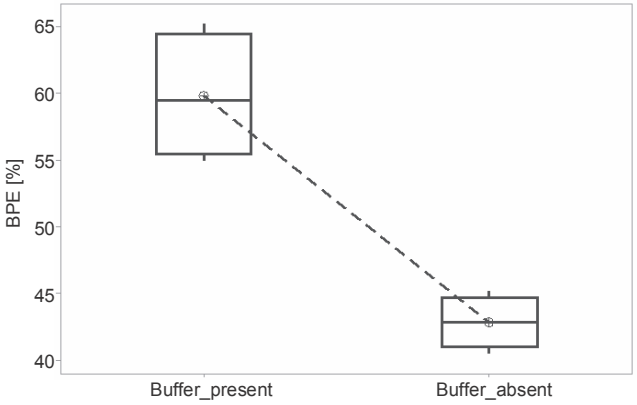

Fig. 4. Box plot showing the effect of acetate buffer on BPE

concentration of 9 g/l and did not improve further. Reducing sugars, 2.85 g/l as glucose equivalents, were still left unutilized in the fermentation broth. This is possibly because the bacterial metabolic pathway could have changed due to the dynamic microenvironment and altered the broth pH after 48 h of fermentation (Duport et al., 2016). In the screening experiments, buffering agents were not used; hence, the change in pH could have affected ethanol production adversely over time. We hypothesize that the use of a suitable buffering system in the fermentation medium could improve bioethanol production. Reportedly, higher concentration of weak acid could be detrimental to bioethanol yield; however, low concentration could show a positive effect (Pampulha and Loureiro-Dias, 1989). In ethanolic fermentation using yeast, Greetham (2015) used acetic acid (<20 mM) in the medium to improve ethanol yield. However, Mols et al. (2010) reported bacteriostatic effect on *B. cereus* grown in minimal medium with 15 mM undissociated acetic acid. To validate this finding, experiments in duplicate were carried out by adding 1 ml of 0.4 M acetate buffer (final 4 mM ionic strength in broth) to POC hydrolysate and adjusting the pH by using aq. NaOH solution. Ethanol yield in terms of BPE of the acetate-buffered production medium was compared with that of nonbuffered production medium as control. All the experiments were conducted with 9 g/l glucose at pH 5.0. When acetate buffer was used, statistical t test showed a significant improvement in the BPE at 1% significance level ($t_{df=3} = 6.63$, $P = 0.007$). The mean BPE and sample variance for the buffered and nonbuffered systems are shown using a box plot (Fig. 4). In *B. cereus*, the metabolic pathway for producing ethanol from glucose is associated with acetate synthesis through acetyl Co-A. With the increase in acetate ion concentration, the genes encoding the enzyme cassette for this pathway are downregulated, leading to a metabolic shift toward ethanol production. Although *B. cereus* can tolerate up to 12 mM undissociated acetate ion concentration, about 5 mM undissociated acetate ion concentration is best recommended for ethanol production (de Sarrau et al., 2012). Thus, the use of acetate buffer in bioethanol production seems to have a beneficial effect and was used for further experiments.

B. cereus (ATCC 14579) can grow on several nitrogen sources and yeast extract. In the POC hydrolysate, organic nitrogen was present in the form of oligopeptides and amino acids (1.95 g/l). Yeast extract was used as a supplementary requirement for organic nitrogen in screening experiments, but the statistical analysis showed a nonsignificant effect of yeast extract on BPE. Hence, yeast extract as an additional nutrient in POC hydrolysate was determined to be unnecessary. Another factor, inoculum size, showed a moderate positive effect of 2.934 on BPE; however, it was nonsignificant at 5% significance level. Inhibitory environment such as toxic substances produced during acid hydrolysis could be compensated by increasing the inoculum size (Pienkos and Zhang, 2009). Hence, in further experiments to optimize the process, higher inoculum size could be used. Other factors such as agitation speed and fermentation temperature showed positive effects (2.220 and 0.345, respectively) but were statistically nonsignificant ($P = 0.211$ and $P = 0.832$) in improving BPE. The positive effect noted here indicates a direct relationship between agitation speed or fermentation temperature and the dependent variable BPE. In a study related to growth conditions of *B. cereus*, de Sarrau et al. (2012) observed that highly controlled growth temperature was unnecessary under anaerobic conditions. However, they observed a significantly lower growth rate and ethanol production at 15°C. Therefore, the following factorial variables were chosen to optimize and improve BPE: glucose concentration, broth pH controlled with acetate buffer, and fermentation duration. Other factors that are nonsignificant to improve BPE were kept constant, viz. temperature at 30°C, agitation speed at 100 rpm, and inoculum size corresponding to $OD_{610} = 1.0$. Yeast extract was excluded from the fermentation medium.

Table 5. Experimental design used in BBD for optimization experiments and mean response (BPE) in each experiment

Standard order	Run order	Factorial level			BPE [%]
		glucose (X_1)	pH (X_2)	time (X_3)	
1	3	−1	−1	0	59.36
2	11	+1	−1	0	88.37
3	14	−1	+1	0	58.32
4	12	+1	+1	0	38.79
5	6	−1	0	−1	43.38
6	8	+1	0	−1	81.93
7	9	−1	0	+1	91.76
8	15	+1	0	+1	92.54
9	4	0	−1	−1	54.57
10	10	0	+1	−1	21.98
11	7	0	−1	+1	78.9
12	2	0	+1	+1	34.87
13	5	0	0	0	91.45
14	1	0	0	0	87.42
15	13	0	0	0	92.27

Process optimization using the Box-Behnken design

A response surface experimental design, namely Box-Behnken design (BBD), with 3 levels was used to optimize glucose concentration, broth pH, and fermentation duration. This design used a lesser number of experiments and provided adequate information to fit a quadratic model to the experimental data. Table 5 provides details on the experimental combinations used for BBD and the response measured as BPE (mean of duplicates). The response (BPE) was fit to a quadratic model, and ANOVA was carried out. Nonsignificant terms were eliminated, and a reduced fit model was developed at 5% significance level. ANOVA table for the reduced fit model (Eq. 4) is given in Table 6. Accuracy of the reduced fit model was statistically verified using a nonsignificant lack-of-fit test ($P = 0.139$) and a high coefficient of determination (92.3%). Not only linear terms, but a few nonlinear (square and interaction) terms were also found to be significant.

$$\text{BPE}\,[\%] = 93.51 + 6.10X_1 - 15.90X_2 + 13.02X_3 - \\ + 32.14X_2X_2 - 13.95X_3X_3 - 12.14X_1X_2 - 9.44X_1X_3 \quad (4)$$

Using D optimal design, the optimal factorial settings to maximize the BPE were determined to be 9 g/l glucose concentration, broth pH 4.8, and 51 h of fermentation time. At this combination, a BPE of 91.8 ± 1.9% and ethanol titer of 4.2 ± 0.2 g/l were experimentally obtained against the theoretically predicted BPE of 104.0 ± 4.4%. Although the experimental BPE was smaller than the theoretical BPE, a statistical *t* test conducted at 5% significance level showed nonsignificant differences between the two mean values. A 90% conversion of reducing sugars (as glucose equivalents) affirmed the results. Thus, the reduced fit model suggested a strong positive effect of glucose concentration, and this effect was enhanced by acidic pH and moderately shorter fermentation period. These effects were also supported by the contour plots (Fig. 5) drawn at optimal conditions.

The *B. cereus* ATCC 14579 strain cannot metabolize the pentose monosaccharide xylose in minimal medium (Mols et al., 2007). However, POC hydrolysate contained very low amounts of xylose equivalents (1.1 g/l) than glucose equivalents (6.9 g/l). Hence, under optimal conditions for batch anaerobic fermentation, a higher conversion of reducing sugars was possible. The ethanol titer was comparatively equal to or better than the reported values of 4 to 5 g/l for *P. pinnata* oil cake (Doshi and Srivastava, 2013; Muktham et al., 2016; Radha-

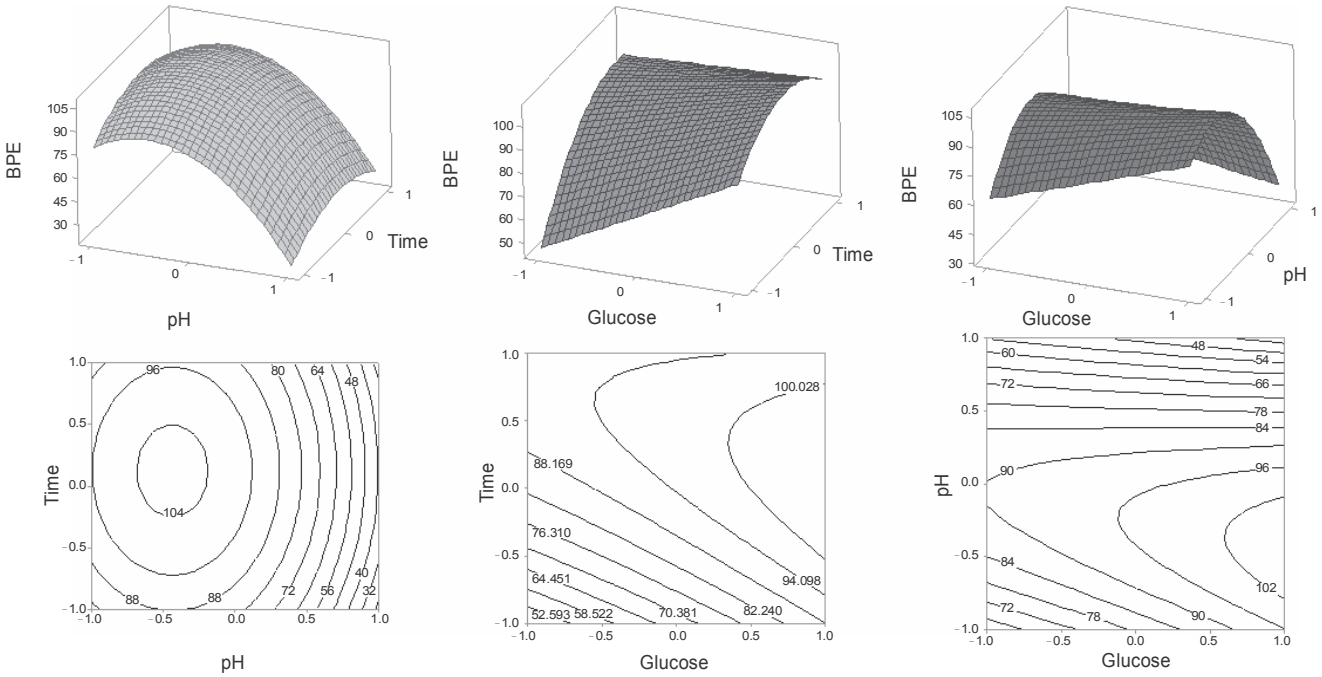

Fig. 5. Contour plots drawn for the reduced fit model at optimal settings; nonlinear variation of BPE is shown with two parameters varied at a time and the third variable is maintained constant at the optimal setting; constant optimal settings: Glu = 9 g/l, pH = 4.6, and fermentation period = 51 h

Table 6. ANOVA table for the reduced fit model obtained through BBD experiments

Source	DF	SS	MS	F	*P*
Model	7	8967.9	1281.1	24.8	< 0.001
Linear	3	3677.4	1225.8	23.8	< 0.001
glucose (X_1)	1	298.0	298.0	5.8	0.047
pH (X_2)	1	2022.4	2022.4	39.2	< 0.001
time (X_3)	1	1357.1	1357.1	26.3	0.001
Square	2	4343.9	2172.0	42.1	< 0.001
pH*pH (X_2X_2)	1	3837.0	3837.0	74.4	< 0.001
time*time (X_3X_3)	1	722.7	722.7	14.0	0.007
2-Way Interaction	2	946.7	473.3	9.2	0.011
glucose*pH (X_1X_2)	1	589.9	589.9	11.4	0.012
glucose*Time (X_1X_3)	1	356.8	356.8	6.9	0.034
Error	7	361.0	51.6		
lack-of-fit	5	340.1	68.0	6.5	0.139
pure error	2	20.9	10.5		
Total	14	9328.9			

kumari et al., 2017). However, in the present study, the BPE improved significantly from the maximum reported value of 43% to 92%. The presence of sugar degradation products such as HMF and furfural and its derivatives could inhibit ethanolic fermentation of cellulosic biomass (Fu et al., 2014; Moreno et al., 2019). The presence of HMF and furfural, each at 1 g/l concentration, can decrease ethanol yield significantly (Fu et al., 2014). In the present study, microwave-assisted sugar hydrolysis produced 350 mg/l HMF and 70 mg/l furfural. A possible

reason for higher BPE could be the low amount of toxic substances produced during microwave-assisted acid hydrolysis. Moreover, the upregulation of the metabolic pathway under anaerobic acidic stress supplemented with acetate ions cannot be overruled. The capability of *B. cereus* to dwell on most of the simple sugars could have compounded this effect. Thus, a bacterial strain that can assimilate majority of sugars and almost completely convert them to ethanol is reported here. *Pongamia* seeds can be used to produce not only biodiesel but also bioethanol, thus making it a viable and economical feedstock for dual fuel production.

Conclusions

By using a facultative anaerobe, *B. cereus*, the production of bioethanol from microwave-assisted sulfuric acid hydrolysate of *Pongamia* oil cake was demonstrated. The parametric analysis using a PBD showed that broth pH, incubation period, and glucose concentration were the significant factors. To improve the BPE and obtain optimal factorial settings, the Box-Behnken experimental design was used. An optimal condition of 9 g/l glucose, broth pH 4.8, and 51 h of fermentation period yielded approximately 92% BPE. Statistical analyses showed a consistency between the predicted results and the experimental results. In addition to controlling pH variations during the fermentation process, acetate buffer significantly improved bioethanol production.

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References

Ahn S.-J., Burne R.A. (2007) *Effects of oxygen on biofilm formation and the AtlA autolysin of Streptococcus mutans.* J. Bacteriol. 189(17): 6293–6302. doi: 10.1128/JB.00546-07.

Al-Showiman S.S. (1998) *Furfural from some decorative plants grown in Saudi Arabia*. J. Sci. Ind. Res. 57: 907–910.

Bradford M. (1976) *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding*. Anal. Biochem. 72(1–2): 248–254. doi: 10.1006/abio.1976.9999

Brown T.R., Brown R.C. (2013) *A review of cellulosic biofuel commercial-scale projects in the United States*. Biofuel. Bioprod. Biorefin. 7(3): 235–245. doi: 10.1002/bbb.1387

Chandrashekar L.A., Mahesh N.S., Gowda B., Hall W. (2012) *Life cycle assessment of biodiesel production from Pongamia oil in rural Karnataka*. Agric. Eng. Int: CIGR J. 14(3): 67–77.

Doshi P., Srivastava G. (2013) *Sustainable approach to produce bioethanol from Karanja (Pongamia pinnata) oilseed residue*. Turk. J. Agric. For. 37: 781–788. doi: 10.3906/tar-1207-18

Duport C., Jobin M., Schmitt P. (2016) *Adaptation in Bacillus cereus: From stress to disease*. Front. Microbiol. 7: 1550. doi: 10.3389/fmicb.2016.01550

Dwivedi G., Jain S., Sharma M.P. (2011) *Pongamia as a source of biodiesel in India*. Smart Grid Renew. Energ. 2(3): 184–189. doi: 10.4236/sgre.2011.23022

Fu S., Hu J., Liu H. (2014) *Inhibitory effects of biomass degradation products on ethanol fermentation and a strategy to overcome them*. Bioresources 9(3): 4323–4335. doi: 10.15376/biores.9.3.4323-4335

Goodwin J.F. (1970) *Method for simultaneous direct estimation of glucose and xylose in serum*. Clinic. Chem. 16(2): 7.

Greetham D. (2015) *Presence of low concentrations of acetic acid improves fermentations using Saccharomyces cerevisiae*. J. Bioprocess. Biotech. 5(1): 192. doi: 10.4172/2155-9821.1000192.

Harreh D., Saleh A.A., Reddy A.N.R., Hamdan S. (2018) *An experimental investigation of Karanja biodiesel production in Sarawak, Malaysia*. J. Engg. 2018: 1–8. doi: 10.1155/2018/4174205

Ire F.S., Ezebuiro V., Ogugbue C.J. (2016) *Production of bioethanol by bacterial co-culture from agro-waste-impacted soil through simultaneous saccharification and co-fermentation of steam-exploded bagasse*. Bioresour. Bioprocess. 3: 26. doi: 10.1186/s40643-016-0104-x

Iyyaswami R., Halladi V.K., Yarramreddy S.R., Malur Bharathaiyengar S. (2013) *Microwave-assisted batch and continuous transesterification of karanja oil: process variables optimization and effectiveness of irradiation: Microwave-assisted transesterification of karanja oil*. Biomass. Conv. Bioref. 3(4): 305–317. doi: 10.1007/s13399-013-0080-8

John I., Pola J., Thanabalan M., Appusamy A. (2019) *Bioethanol production from Musambi peel by acid catalyzed steam pretreatment and enzymatic saccharification: Optimization of delignification using Taguchi design*. Waste Biomass Valor. Accepted Manuscript. doi: 10.1007/s12649-018-00565-x

Kamath V.H., Suvarna D.D., Drathi U.K., Gayathri J., Rao C.V. (2018a) *Microwave accelerated dilute acid hydrolysis of Pongamia pressed oil cake for release of fermentable sugars*. Adv. Biores. 9(6): 109–118. doi: 10.15515/abr.0976-4585.9.6.109118

Kamath H.V., Shenoy A.G., Crasta I., Rao S., Rao C.V. (2018b) *Microwave assisted hydrolysis of cellulose to release sugars from Pongamia oil cake for its use in bioethanol production*. Chem. Sci. Trans. 7(4): 722–728. doi: 10.7598/cst2018.1557

Lopes M.L., Paulillo S.C. de L., Godoy A., Cherubin R.A., Lorenzi M.S., Giometti F.H.C., Bernardino C.D., Amorim

Neto H.B. de, Amorim H.V. de (2016) *Ethanol production in Brazil: a bridge between science and industry*. Brazil J. Microbiol. 47: 64–76. doi: 10.1016/j.bjm.2016.10.003

Miller G.L. (1959) *Use of dinitrosalicylic acid reagent for determination of reducing sugar*. Anal. Chem. 31(3): 426–428. doi: 10.1021/ac60147a030

Mishra R.K., Mohanty K. (2018) *Characterization of non-edible lignocellulosic biomass in terms of their candidacy towards alternative renewable fuels*. Biomass Conv. Bioref. 8(4): 799–812. doi: 10.1007/s13399-018-0332-8

MNRE (2018) *National Policy on Biofuels – 2018*. Ministry of Petroleum & Natural Gas, Govt. of India.

Mols M., Abee T. (2011) *Bacillus cereus responses to acid stress*. Env. Microbiol. 13(11): 2835–2843. doi: 10.1111/j.1462-2920.2011.02490.x

Mols M., de Been M., Zwietering M.H., Moezelaar R., Abee T. (2007) *Metabolic capacity of Bacillus cereus strains ATCC 14579 and ATCC 10987 interlinked with comparative genomics*. Environ. Microbiol. 9(12): 2933–2944. doi: 10.1111/j.1462-2920.2007.01404.x

Mols M., Van Kranenburg R., Van Melis C.C.J., Moezelaar R., Abee T. (2010) *Analysis of acid-stressed Bacillus cereus reveals a major oxidative response and inactivation-associated radical formation: acid-induced radical formation in B. cereus*. Env. Microbiol. 12(4): 873–885. doi: 10.1111/j.1462-2920.2009.02132.x

Moreno A.D., Carbone A., Pavone R., Olsson L., Geijer C. (2019) *Evolutionary engineered Candida intermedia exhibits improved xylose utilization and robustness to lignocellulose-derived inhibitors and ethanol*. App. Microbiol. Biotechnol. 103(3): 1405–1416. doi: 10.1007/s00253-018-9528-x

Muktham R., Ball A.S., Bhargava S.K., Bankupalli S. (2016) *Bioethanol production from non-edible de-oiled Pongamia pinnata seed residue-optimization of acid hydrolysis followed by fermentation*. Ind. Crop Product. 94: 490–497. doi: 10.1016/j.indcrop.2016.09.019

Namadi M.M. (2013) *Evaluation of sugar content and bioethanol potentials of some freshwater biomass*. I. J. Renew. Sustain. Energ. 2(6): 201. doi: 10.11648/j.ijrse.20130206.12

Omidvar M., Karimi K., Mohammadi M. (2016) *Enhanced ethanol and glucosamine production from rice husk by NAOH pretreatment and fermentation by fungus Mucor hiemalis*. Biofuel Res. J. 3(3): 475–481. doi: 10.18331/BRJ2016.3.3.7

Pampulha M.E., Loureiro-Dias M.C. (1989) *Combined effect of acetic acid, pH and ethanol on intracellular pH of fermenting yeast*. App. Microbiol. Biotechnol. 31–31(5–6): 547–550. doi: 10.1007/BF00270792

Passoth V., Sandgren M. (2019) *Biofuel production from straw hydrolysates: current achievements and perspectives*. App. Microbiol. Biotechnol. 103(13): 5105–5116. doi: 10.1007/s00253-019-09863-3

Pienkos P.T., Zhang M. (2009) *Role of pretreatment and conditioning processes on toxicity of lignocellulosic biomass hydrolysates*. Cellulose 16(4): 743–762. doi: 10.1007/s10570-009-9309-x

Radhakumari M., Taha M., Shahsavari E., Bhargava S.K., Satyavathi B., Ball A.S. (2017) *Pongamia pinnata seed residue – a low cost inedible resource for on-site/in-house lignocellulases and sustainable ethanol production*. Renew. Energ. 103: 682–687. doi: 10.1016/j.renene.2016.10.082

de Sarrau B., Clavel T., Clerté C., Carlin F., Giniès C., Nguyen-The C. (2012) *Influence of anaerobiosis and low temperature on Bacillus cereus growth, metabolism, and membrane properties*. App. Env. Microbiol. 78(6): 1715–1723. doi: 10.1128/AEM.06410-11

Schell D.J., Dowe N., Chapeaux A., Nelson R.S., Jennings E.W. (2016) *Accounting for all sugars produced during integrated production of ethanol from lignocellulosic biomass*. Bioresour. Technol. 205: 153–158. doi: 10.1016/j.biortech.2016.01.024

Shahirah M.N.N., Gimbun J., Pang S.F., Zakria R.M., Cheng C.K., Chua G.K., Asras M.F.F. (2015) *Influence of nutrient addition on the bioethanol yield from oil palm trunk sap fermented by Saccharomyces cerevisiae*. J. Ind. Eng. Chem. 23: 213–217. doi: 10.1016/j.jiec.2014.08.018

Sindhu R., Binod P., Mathew A.K., Abraham A., Pandey A., Gnansounou E., Castro G.E. (2018) *An effective surfactant-assisted hydrothermal pretreatment strategy for bioethanol production from chili post-harvest residue by separate hydrolysis and fermentation*. Bioprocess. Biosyst. Eng. 41(4): 565–571. doi: 10.1007/s00449-018-1891-6

Singh S., Adak A., Saritha M., Sharma S., Tiwari R., Rana S., Arora A., Nain L. (2017) *Bioethanol production scenario in India: potential and policy perspective*. [in:] *Sustainable Biofuels Development in India*. Ed. Chandel A.K., Sukumaran R.K. Cham, Springer International Publishing: 21–37.

Sunwoo I., Kwon J.E., Nguyen T.H., Jeong G.-T., Kim S.-K. (2019) *Ethanol production from water hyacinth (Eichhornia crassipes) hydrolysate by hyper-thermal acid hydrolysis, enzymatic saccharification and yeasts adapted to high concentration of xylose*. Bioprocess Biosyst. Eng. 42(8): 1367–1374. doi: 10.1007/s00449-019-02136-3

White J.W. (1979) *Spectrophotometric method for hydroxymethylfurfural in honey*. J. Assoc. Off. Anal. Chem. 62(3): 509–514.