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Production of pullulan by *Aureobasidium pullulans* from Asian palm kernel: A novel substrate

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ABSTRACT

Production of a commercially important biodegradable polymer, pullulan, by *Aureobasidium pullulans* from four agricultural wastes namely wheat bran, rice bran, coconut kernel and palm kernel was evaluated in solid state fermentation. Under the experimental conditions, palm kernel resulted in highest concentration of pullulan (16 g/L) among the four solid substrates. Optimum initial pH and moisture content for pullulan production were found out to be 6.5 and 50% respectively. 18.43 g/L of pullulan was produced from Asian palm kernel with initial pH 6.5 after 7 days of fermentation and yeast like morphology was predominant under this condition. Among different nitrogen sources tried in this study, yeast extract was found to the best. The pullulan produced from palm kernel was characterized by FTIR and ^{1}H NMR. The results were matching with that of commercial pullulan. Thus, Asian palm kernel appears to be an attractive low cost carbon source for the production of pullulan.

1. Introduction

Pullulan is a linear, un-branched exo-polysaccharide (Carolan, Catley, & McDougal, 1983; Taguchi, Kikuchi, Sakano, & Kobayashi, 1973) which consists of uniform linkage pattern of maltotriose units which are attached by an α (1 → 4) glycosidic linkages and repeated maltotriose units which are attached to each other by an α (1 → 6) glycosidic linkages (Catley, Ramsay, & Servis, 1986; Sutherland, 1998). It is a water soluble biopolymer but it is insoluble in organic solvents, excluding dimethylformamide and dimethylsulfoxide (Leathers, 2002; Sugimoto, 1978). It is nontoxic, tasteless, odourless, white, and non-hygroscopic (Leathers, 2002; Sugimoto, 1978).

Aureobasidium pullulans called as black yeast have five different cell morphologies like swollen blastospores, yeast-like cells, mycelia, chlamydospores and young blastospores (Ronen, Guterman, & Shabtai, 2002). From an ecological point of view, *A. pullulans* is mainly found on leaves and various surfaces such as concrete, lime stone, wood, soil and forest barks (Bhadra, Rao, Singh, Sarkar, & Shivaji, 2008). *A. pullulans* is a polymorphic fungus, ranging from blastic conidia and swollen cells to pseudohyphae, hyphae, and chlamydospores, depending upon age of inoculmn, culture conditions and medium composition (Leathers, 2003) that produces pullulan (Gibbs & Seviour, 1992).

Recently pullulan has been widely used in pharmaceutical industry as a biomaterial (Alban, Schauerte, & Franz, 2002; Masci, Bontempo, & Crescenzi, 2002; Sivakumar & Rao, 2003). Pullulan can be also used as a non-caloric food ingredient, dietary food as a starch. It forms transparent film, impervious to oxygen transfer and used as a packing and coating materials in food and pharmaceutical industries (Deshpande, Rale, & Lynch, 1992). Pullulan is widely used in high performance liquid chromatography (HPLC) columns and in size exclusion chromatography as a molecular mass standard (Buliga & Brant, 1987).

In spite of its commercial importance and wide spread applications in the fields of food, pharmaceutical, lithography and other fields, its use in large scale is limited by the economic constraints. In the recent review on the pullulan, it is reported that the cost of pullulan is about three times higher than that of other polysaccharides (Ram, Gaganpreet, & Kennedy, 2008). Various approaches had been adopted to bring the cost pullulan production. This includes, engineering innovations, improved strains (Ram et al., 2008) and identification of cheaper and effective carbon and nitrogen sources (Wu, Jin, Tong, & Chen, 2009; Göksungur, Uzunoğulları, & Dağbağlı, 2011). It had been reported that cost of the media components accounts for 30% of total production cost (Miller & Churchill, 1986; Nishat Sharma, Prasad, & Choudhury, in press). Agro-industrial wastes (Israilides, Bocking, Smith, & Scanlon, 1994, Israilides et al., 1998),

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potato starch waste (Barnett, Smith, Scanlon, & Israilides, 1999), deproteinized whey (Roukas, 1999a), brewery wastes (Roukas, 1999b), jaggery (Vijayendra, Bansal, Prasad, & Nand, 2001), beet molasses (Lazaridou, Roukas, Biliaderis, & Vaikousi, 2002), sweet potato (Wu et al., 2009), coconut by-products (Thirumavalavan, Manikkandan, & Dhanasekar, 2009), hydrolyzed potato starch waste (Göksungur et al., 2011), and corn steep liquor (Nishat Sharma et al., in press) were the alternate carbon sources reported in pullulan literature.

In this study, four different agricultural wastes namely wheat bran, rice bran, coconut kernel and palm kernel were evaluated as possible low cost carbon sources for the production of microbial pullulan. Asian palm kernel emerged to be the best carbon source among the four. The effects of moisture content, nitrogen source, initial pH and fermentation time on pullulan production from Asian palm kernel were investigated in solid state fermentation. To the best of our knowledge, this is the first report on production of pullulan by *A. pullulans* in solid state fermentation using palm kernel as a sole carbon source.

2. Materials and methods

2.1. Microorganisms and culture conditions

A. pullulans MTCC 2670 was purchased from MTCC, Chandigarh, India. Stock cultures of the fungi were maintained on potato dextrose agar at 4 °C and sub cultured every 3 weeks. Potato dextrose agar composition was: potato (scrubbed and diced) – 200 g/L; dextrose – 20 g/L; agar – 15 g/L.

2.2. Inoculum preparation in culture medium

Basal medium contains the following: sucrose – 30 g/L; $(NH_4)_2SO_4$ – 2 g/L; yeast extract – 0.4 g/L; K_2HPO_4 – 5.0 g/L; $MgSO_4{\cdot}7H_2O$ – 0.2 g/L and NaCl – 1.0 g/L and pH was adjusted to 7 before sterilization. The medium was sterilized for 15 min at 121 °C and cooled. Two loops of *A. pullulans* cells were transferred to 250 mL Erlenmeyer flasks containing 50 ml of sterilized culture medium which was incubated at 30 °C for 48 h in an orbital shaker at 200 rpm. These cultures were used as inoculum for pullulan production.

2.3. Solid state fermentation

Palm kernel was used as solid substrate and carbon source in solid state fermentation. Palm kernel was obtained from matured palmyra fruit (*Borassus flabellifer*). The outer most layers of the palmyra fruits were removed and the matured inner part (palm kernel) of the palmyra fruits had been cut into pieces. These pieces were sun-dried for 6–8 days to prevent microbial deterioration. Solid substrate was prepared by taking 20 g of palm kernel pieces in 250 mL Erlenmeyer flask and the volume of basal medium was varied according to the moisture content. The basal medium was added with solid substrate and initial pH was adjusted to 7.5 (before sterilization). Then mixture was sterilized for 15 min at 121 °C and sterilized medium was inoculated with 2% v/v of 48 h old culture (0.8 O.D at 650 nm) of *A. pullulan* grown on culture medium.

2.4. Determination of the effect of different factors on pullulan production

As mentioned earlier in the introduction section, four different agro-wastes namely wheat bran, rice bran, coconut kernel and palm kernel were examined for the production of microbial pullulan. After screening solid substrate, effects of these variables such as initial pH (3–11), fermentation time (1–8 days), initial moisture content (10–90%) and screening of nitrogen sources such as ammonium sulfate, ammonium chloride, peptone, yeast extract and malt extract on pullulan production were studied.

2.5. Estimation of pullulan concentration

Samples were taken from fermentation medium and centrifuged at 10,000 × *g* for 20 min for estimating pullulan concentration. The supernatant obtained from centrifugation was precipitated by adding two volumes of ethanol at 4 °C for 1 h. Then precipitate was treated with acetone and filtered by pre-weighed Whatman No.1 filter paper and dried at 90 °C for constant weight and dry weight of pullulan was expressed as g/L (Vijayendra et al., 2001).

2.6. Morphological observation and yeast biomass estimation

Cell morphology was observed using light microscopy (Carl Zeiss inc. Germany). Estimation of yeast biomass in dry weight was carried out according to the method described by Reeslev, Nielsen, Olsen, Jensen, and Jacobsen (1991). Sample was taken from solid state fermentation under aseptic condition and centrifuged at 12,000 rpm for 10 min centrifugation was carried out three times for efficient separation of cells and substrate from supernatant. It was ensured that cells were not present in the supernatant using light microscope (Carl Zeiss inc., Germany). The pellet so collected was added with 5 mL of 1% NaCl solution. Then sample was filtered through a nylon mesh of 41 μm square porosity for the separation of yeast cells from mycelium and solid substrate (Reeslev et al., 1991). Using light microscope, it was conformed that only yeast like cells were appeared in the filtrate and yeast like cells were dried at 90 °C to constant weight. Mycelia mat on the mesh and solid substrate were washed with water and dried at 90 °C to constant weight. Yeast biomass was expressed by percentage of total dry weight of solid substrate and cells (Mitchell, Krieger, & Berovi, 2006).

2.7. Characterization of pullulan

The purified sample was characterized using IR spectroscopy (Perkin-Elmer 1600 spectrophotometer) and ^1H NMR (Bruker 300 MHz Instrument, Germany) and results were compared with that of commercial pullulan (TCI chemicals, Tokyo). FTIR sampling was done with KBr pellet method (Hui-zhu et al., 2009; Thirumavalavan et al., 2009) and ^1H NMR sample was prepared by dissolving 10 mg of pullulan in 0.5 mL DMSO-d_6 solvent and TMS was used as an internal standard (Hui-zhu et al., 2009).

3. Result and discussion

3.1. Screening of solid substrate

Fig. 1 shows the screening of solid substrate on pullulan production with 50% moisture content. Maximum pullulan concentration obtained with these carbon sources were 5.5, 7.5, 9.5 and 16.0 g/L for rice bran, wheat bran, coconut kernel and palm kernel respectively. Therefore, palm kernel was selected as a solid substrate for pullulan production and used for further studies. Thirumavalavan et al. (2009) obtained pullulan concentration of 38.3 g/L and 58 g/L from coconut water and coconut milk respectively in submerged fermentation. Vijayendra et al. (2001) had reported 51 g/L pullulan yield from jaggery and derivative of sucrose. Ray and Moorthy (2007) produced pullulan from wheat bran, rice bran and cassava starch with and without basal medium and maximum production of pullulan 27.5 g/kg of substrate, was achieved using cassava starch residue as a solid substrate (Ray & Moorthy, 2007).

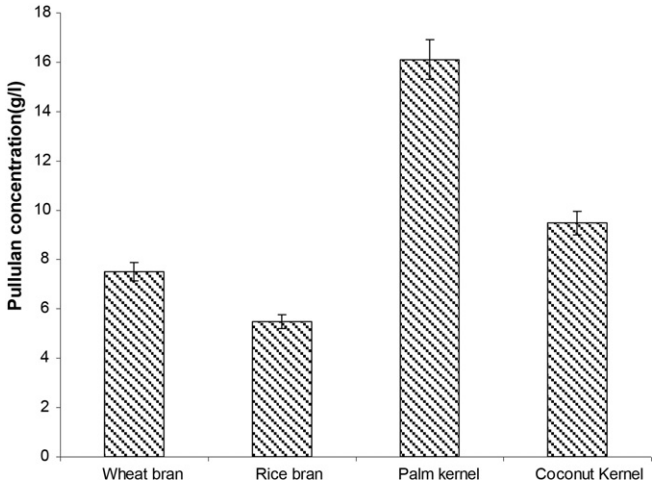

Fig. 1. Screening of solid substrate for pullulan production (±5% error bar).

3.2. Effect of initial pH on morphology and pullulan concentration

pH is a crucial factor affecting the morphology of *A. pullulan* as well as pullulan production (Goksungur, Ucan, & Guvenc, 2004; Seviour, Kristiansen, & Harvey, 1984). Fig. 2 explains the effect of pH on pullulan production utilizing palm kernel as a solid substrate and 50% moisture content was maintained in the fermentation medium before sterilization. Pullulan concentration gradually increased with increase in initial pH up to a pH of 7. However, pullulan concentration decreased with further increase in pH. Low pH favours the filamentous morphology whereas high pH favours the yeast like morphology in solid state fermentation. Yeast dry biomass (% of total dry weight of sample) was kept on increasing with increase in initial pH in the fermentation medium. Maximum concentration of pullulan (16.39 g/L) was obtained when yeast dry biomass was found to be maximum (33% of total dry weight of sample). Light microscopic images taken at different initial pH are shown in Fig. S1 (supplementary file). Yeast like morphology containing more swollen cells was predominant in the fermentation medium at initial pH of 6.5 which enhance the pullulan production. This is consistent with the earlier finding that yeast like morphology of *A. pullulan* is responsible for the production of pullulan (Heald & Kristiansen, 1985; Ronen et al., 2002). Heald and Kristiansen (1985) had reported that maximum pullulan concentration was achieved at pH of 6.3 with 60% yeast like morphology in the fermentation system. Harvey (1984) had shown that yeast like structure was predominant at pH 6, while filamentous forms were dominant at low

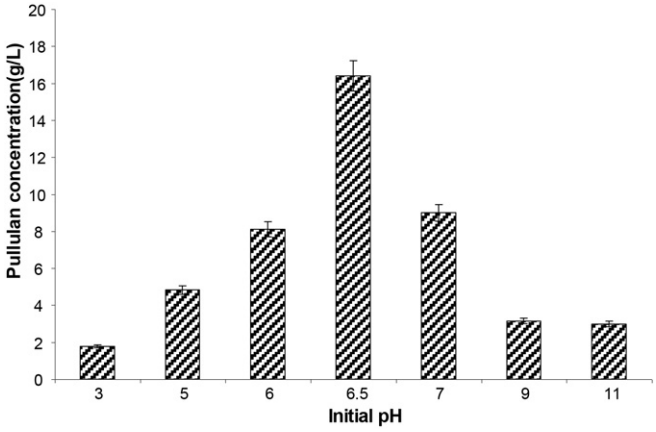

Fig. 2. Effect of initial pH on pullulan production (±5% error bar).

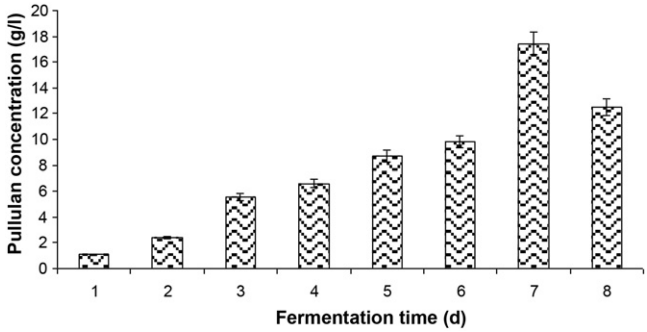

Fig. 3. Effect of fermentation time on pullulan production (5% error bar).

pH 2.5. It has been that swollen cells, blastospores and chlamydospores were important cellular constituents responsible for pullulan production (Campbell, Siddique, McDougall, & Seviour, 2004; Catley, 1980; Li et al., 2009; Simon, Bouchet, & Caye-Vaugien, 1995).

Catley (1971) first explained the effect of pH on pullulan production. Similar kinds of studies were carried out by several investigators. Thirumavalavan et al. (2009) reported that at initial pH 7, high concentration of pullulan was obtained from coconut milk (Thirumavalavan et al., 2009). The suitable pH for pullulan production has been explained between 5.5 and 7.5 by several researchers (Cheng, Demirci, & Catchmark, 2009; Lee & Yoo, 1993; Shingel, 2004). Pullulan production was high as initial pH was at pH 5.5 (Lacroix, LeDuy, Noel, & Choplin, 1985). Polysaccharide concentration was maximum using carob pod extract at an initial pH of 6.5 (Israilides et al., 1998) while using synthetic medium, maximum polysaccharide concentration was achieved at initial pH 6 (Ono, Kawahara, & Ueda, 1977).

3.3. Effect of fermentation time

In this study, sample was withdrawn from the medium at regular 24 h and pullulan concentration was estimated as described in section 2.5. Fig. 3 explains the effect of fermentation time on pullulan production using palm kernel as a carbon source with an initial pH of 6.5 and 50% initial moisture content was maintained on solid substrate before sterilization. Increase in fermentation time increases the pullulan concentration gradually and reaches a maximum value of 18.43 g/L after a period of seven days. The production begins to decrease during later stages due to the hydrolysis of pullulan by the action of endogenous glucoamylase-A (Thomas & Strohfus, 1996). High pullulan concentration of 54 g/L was obtained from coconut milk for a period of 144 h (Thirumavalavan et al., 2009).

3.4. Effect of initial moisture content

Fig. 4 shows the variation of moisture content ranging from 10% and 90% on pullulan production. The pullulan concentration was relatively low when initial moisture content was low. Pullulan production was increased with increase in initial moisture content and reached maximum level of 17 g/L at 50% moisture content. Further increase in initial moisture content resulted in low pullulan yield.

3.5. Effect of nitrogen source

Polysaccharide formation by *A. pullulans* is strongly influenced by the nitrogen sources in the medium (Auer & Seviour, 1990; Goksungur et al., 2004). The experiments were carried out by varying the nitrogen source such as, ammonium sulfate, ammonium chloride, peptone, malt extract and yeast extract. Maximum production of pullulan was obtained with yeast extract (16.85 g/L).

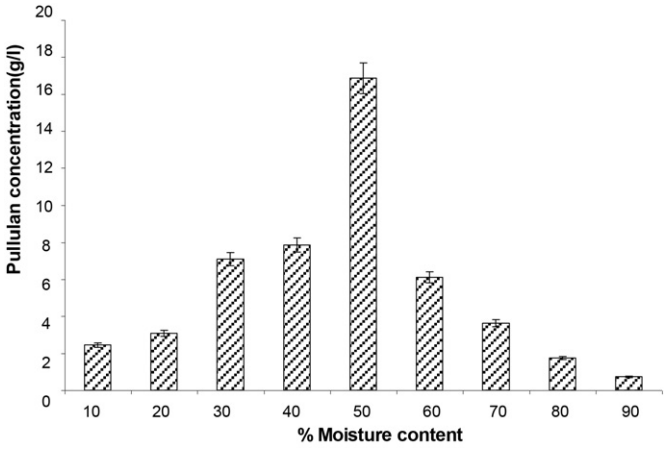

Fig. 4. Effect of moisture content on pullulan production (±5% error bar).

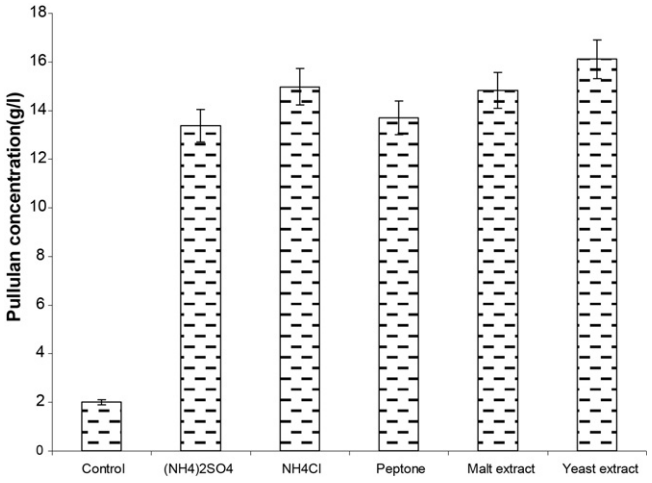

Fig. 5. Screening of nitrogen source on pullulan production (±5% error bar).

However, difference in pullulan production with these nitrogen sources was not significant (Fig. 5).

Several researchers had earlier studied effect of nitrogen source on pullulan production (Auer & Seviour, 1990; Lazaridou et al., 2002; Reed-Hamer & West, 1994; Schuster, Wenzig, & Mersmann, 1993; Seviour, Stasinopouslos, Auer, & Gibbs, 1992; Thirumavalavan et al., 2009). Reed-Hamer and West (1994) reported role of nitrogen sources such as corn steep liquor, peptone, tryptone, casamino acid and soytone on pullulan production using sucrose or corn syrup as a carbon source and concluded that high concentration of pullulan was achieved using soytone as nitrogen source (Reed-Hamer & West, 1994). Auer and Seviour (1990) had described the role of nitrogen sources on pullulan concentration and found NH_4NO_3 and $(NH_4)_2SO_4$ as the best nitrogen sources for pullulan production (Auer & Seviour, 1990). Thirumavalavan et al. (2009) had reported up to 58.6 g/L of pullulan production from coconut milk by *A. pullulans* using yeast extract as a nitrogen source.

3.6. Structural characterization of pullulan

Fig. 6 shows the FT-IR spectra for pullulan from palm kernel. A broad band appeared at 3444 cm^{-1} was due to —OH stretching and a sharp band at 2924 cm^{-1} was characteristic of C—H stretching. Further characteristic signals arrived at 1456, 1140, 1037 and 669 cm^{-1} were due to C—O—H bending, C—O—C stretching, C—O stretching and alpha configuration respectively. A band appeared at 1639 cm^{-1} is coinciding with that of the previous report for vibrations of the C—O—C bond and glycosidic linkage (Kačuráková, Capek, Sasinková, Wellner, & Ebringerová, 2000). The comparison of characteristic absorption frequencies between isolated and commercial samples is listed in Table 1. (Seo, Chung, Jung, Kim, & Gross, 2004; Singh & Saini, 2008).

Table 1
Comparison of FTIR data of isolated and commercial pullulan.

Assignments	Commercial Pullulan wave number (cm^{-1})	Pullulan from palm kernel wave number (cm^{-1})
O—H stretching	3435	3444
C—H stretching	2937	2924
O—C—O stretching	1630	1639
C—O—H bending	1363	1456
C—O—C stretching	1158	1140
C—O stretching	1024	1037

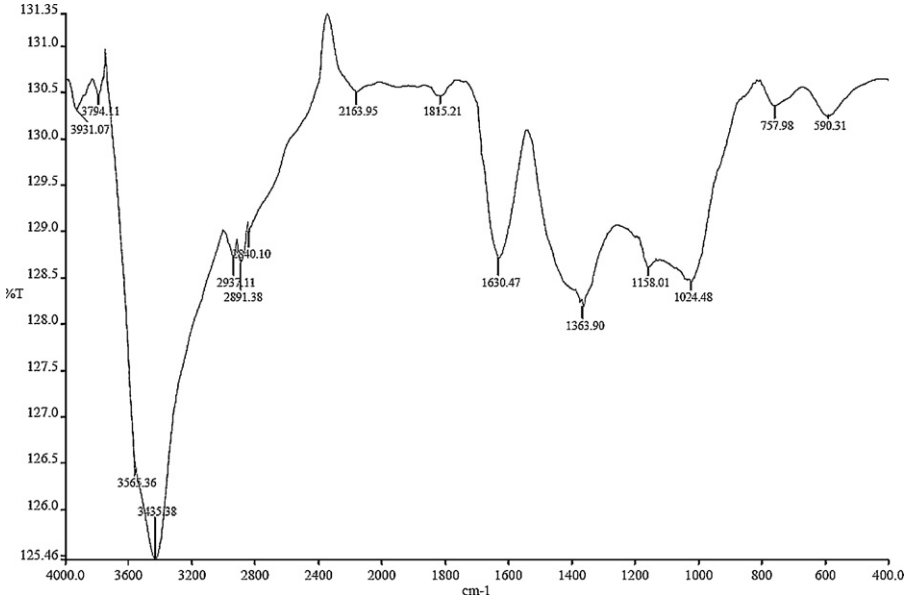

Fig. 6. FTIR for pullulan from palm kernel.

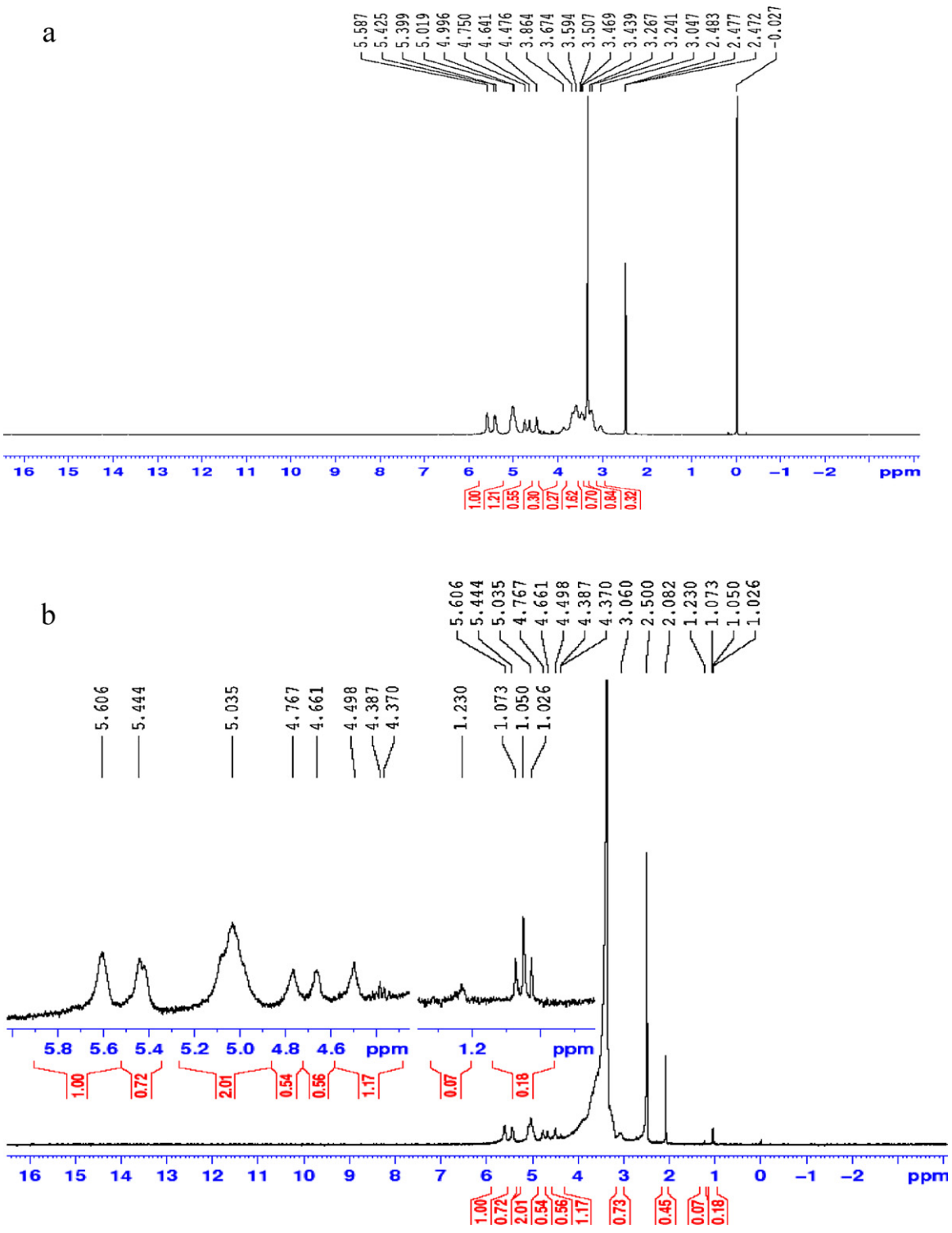

Fig. 7. (a) ^{1}H NMR for commercial pullulan; (b) ^{1}H NMR for pullulan from palm kernal.

Fig. 7(a) and (b) shows the proton NMR for commercial pullulan and palm kernel pullulan. Structural characterization of the purified pullulan from *A. pullulans* was carried out by NMR spectroscopy and it was compared with proton NMR spectra for commercial pullulan. One dimensional proton NMR spectra of the sample exhibits signals integrated for the total of 21 protons. The signals arrived in the downfield region between 4 and 6 ppm infers proton carrying carbon atoms attached to an electronegative atom – a characteristic chemical environment of a carbohydrate moiety. Total integration for 21 protons infers that the repeating unit of the carbohydrate polymer contains 3 mono saccharide units. On the basis of comparison of the signals with that of commercial pullulan spectra, the compound was identified and confirmed as pullulan (Hui-zhu et al., 2009; Ram, Gaganpreet, & Kennedy, 2009).

4. Conclusion

Four agro wastes namely, wheat bran, rice bran, coconut kernel and palm kernel were evaluated as a low carbon source for the pullulan production by *A. pullulans* in solid state fermentation at

50% moisture content. Palm kernel emerged to be the best carbon source among the four agro wastes and yield 16 g/L pullulan. Effects of initial pH, fermentation time and moisture content on pullulan production were investigated. Screening of nitrogen source on pullulan production was done. From this study, it was found that the optimum conditions for pullulan production using palm kernel as a solid substrate in solid state fermentation are: initial pH – 6.5; fermentation time – 7 days, moisture content – 50% and nitrogen source – yeast extract. Microbial pullulan obtained in this study was characterized by FT-IR and ^{1}H NMR. The spectra of the product were matching with that of commercial pullulan. Thus, palm kernel appears to be a potential low cost carbon source for the production of the pullulan.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.09.062.

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