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Review

Application of cassava harvest residues (*Manihot esculenta* Crantz) in biochemical and thermochemical conversion process for bioenergy purposes: A literature review

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Bioenergy production from biomass and agricultural wastes has gained significant interest due to rising fossil fuel prices and their decrease in air pollutant emissions. This review paper evaluates the state-of-art for the several applications from cassava harvest residues and their use in bioenergy industry, using different thermochemical and biochemical processes. Regarding the great available literature for this biomass, several pretreatment techniques, including mechanical, chemical, biological, thermal, ultrasonic and wet explosion were observed. The use of cassava harvest residues for the biochemical pretreatments, for example, hydrolysis, fermentation and thermochemical processes, such as direct combustion, gasification, pyrolysis, fast pyrolysis and oxy-fuel combustion was also discussed. Therefore, studies are necessary in order to understand that the use of cassava residues in thermal processes can increase the viability of this feedstock for biofuels production and/or in power co-firing units. After extensive study, it was observed that informations are still lacking about the use of cassava harvest residues in other conversion processes, thus, new studies to discover more on the use of this biomass, in order to extend their application in the bioenergy market is encouraged.

Key words: Biomass, cassava, harvest, processes, residues, biochemical, thermochemical.

INTRODUCTION

The main issues faced by many developed and developing countries around world are actually the future energetic security and inadequate use of natural resources (Naqvi et al., 2018; Ferreira-Leitão et al., 2010). The geopolitical, environmental and economic scenario requires the urgent development of renewable

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energy resources and, in particular, bioenergy, for example, biomasses (Welfle, 2017).

Biomasses are found abundantly in nature and can be conveniently generated in most non-urban configurations (Ozturk et al., 2017). Generally they are classified into two types: natural materials and derivatives (Toklu et al., 2010). Biomass resources are subdivided into some categories: agricultural production wastes, energy crops, agriculture processing, urban organic, urban pruning-woods and woods mill (Nansaior et al., 2013; Main-Knorn et al*.,* 2013). Conversion of biomass into energy is an alternative that will mitigate negative socioenvironmental impacts, such as rural unemployment and global warming (Mckendry, 2002; Okudoh et al*.*, 2014; Ozturk et al., 2017; Long et al., 2013). Bioenergy from biomass is a clean technology, safe and renewable resource, and is considered as a potential alternative to partially replace fossil fuels, which will decrease in the future (Ali et al., 2017).

Agroindustrial wastes derived from the crop harvest and food processing are examples of renewable resources and can be used as feedstock for generating bioenergy (Simangunsong et al., 2017; Pereira and Costa, 2017). In 2011, Food and Agriculture Organization (FAO) estimated in their annual report that approximately one-third of all food produced for human consumption worldwide is discarded, representing about 1.3 billion metric tons of wastes per year (Kreuger et al*.,* 2011). Despite the large amount of agricultural wastes generated worldwide, their use as biofuel is still irrelevant, mainly due to limited information on its thermochemical characteristics (Ion et al., 2013).

The traditional use of lignocellulosic biomass was by many years limited to burning for cooking and heating, which lead to significant negative environmental impacts such as land degradation and desertification (Lynd et al., 2015). By means of thermochemical and/or biochemical conversion routes, the lignocellulosic biomass can be converted into energy or bioenergy transporters. Thermochemical conversion uses thermal and chemical processes for producing energy products from biomass, including combustion, pyrolysis, oxy-fuel combustion, gasification and liquefaction (Goyal et al., 2008; Cruz and Crnkovic, 2016; Cai et al., 2017).

The bioenergy production from biomass or agricultural wastes has gained significant interest also due to rising fossil fuel prices (Pereira and Costa, 2017). Several studies have determined the physicochemical characteristics of crop residues, such as corn cobs and straws, rice and coffee husks, pine sawdust, olive and *tucumã* seeds, sugarcane bagasse among others (Graham et al., 2007; Donaldson et al., 2001; Berndes et al., 2003; Ezui et al*.*, 2015; Cruz et al., 2017; Veiga et al., 2016). However, detailed information on the use of cassava harvest residues for the different energetic applications is still missing.

Cassava is a perennial plant of the genus *Manihot esculenta* Crantz. The main producer countries of cassava in the world are Nigerian, Brazil, Thailand and Ghana, in that order (Suttibak et al., 2012). Cassava is a shrub cultivated extensively as an annual crop in tropical and subtropical regions, and their root is an edible starchy tube (Edhirej et al., 2017). Their residues are available in the fields after harvest (Zhang et al., 2003). The roots are collected and transported, while some stems are used for crop replating and most of the green mass is left in soil, which decompose and some nutrients return to the soil (Isahak et al., 2012; Sorapipatana and Yoosin*,* 2011; Liu et al., 2013; Sánchez et al*.*, 2017).

It was noted that few papers discussed the use of cassava residues by thermochemical processes as energy source. Pattiya (2011) characterized the cassava wastes used as fuel in Thailand and classified the stalks and seed stem as residues, characterizing them physically and chemically. Wei et al. (2015) discussed the possibility of extracting starch from cassava branches for producing ethanol and also evaluated aspects such as the production origin region. Veiga et al. (2016) sought to quantify and characterize cassava harvest residues by thermogravimetric analysis in oxidizing and inert atmospheres for studying the residues behavior as biofuel.

Due to several factors earlier reported, this review paper is justified for allowing compilation of works that demonstrate the importance of the characterization from cassava harvest residues and the use in bioenergy industry. Regarding the available literature, it was observed that several pretreatment techniques, including mechanical, chemical, biological, thermal, ultrasonic and wet explosion can be employed for this biomass. The use of cassava harvest residues for thermochemical processes was also discussed (direct combustion, gasification, pyrolysis, fast pyrolysis and oxy-fuel combustion).

CHARACTERISTICS OF THE CASSAVA

What is cassava?

Cassava *(Manihot esculenta* Crantz) is a tubercle, 5 to10 cm in diameter and 15 to 35 cm in length (Figure 1). It is cultived in almost all tropical countries and grows in degraded soils, where no other crop can grow (Kuiper, 2007). Furthermore, cassava can be harvested anytime between 8 and 24 months after planting (DAFF, 2010; Okudoh et al*.,* 2014). Regarding cassava starch, this has several industrial applications and creates a huge global business. The raw material is manioc roots. The starch content in the cassava roots varies from 20 to 32% and depends of the region, climate, soil type and crop, while water content in the cassava roots is about 60% (Chavalparit and Ongwandee, 2009; Kristensen et al., 2013).

Figure 1. Cassava tubers. **(A)** Root with stems attached. **(B)** Root without stems attached
Source: Okudoh et al. (2014).

Dry cassava pulp, a residue from starch production,contains around 50% of this polysaccharide and 43% insoluble dietary fiber (dry weight basis). Such pulp when discarded in inappropriate places causes damage to the environment and diseases proliferation in humans transmitted by animals (Tan et al., 2017). Authors reported recovering of the starch via sonication or enzymatic hydrolysis of their fibrous content, using a multi-enzyme mixture of cellulase and pectinase (Agyepong and Barimah, 2017). The pulp, also called cassava fibrous wastes or bagasse, contains between 30 and 50% starch content (dry weight basis) and cellulose and hemicellulose levels of 24.99 and 6.67% (w/w), respectively (Sriroth et al., 2000). After the removal of the tuberous roots, the cassava crop residues and plant shoots are estimated from 144 to 257%. Use of cassava stems, leaves as forage or addition of roots wastes to prepare feed flour is justified, due to their nutritional value and high forage yield per hectare (Bose and Martins Filho, 1984). Several steps are involved in cassava roots processing to obtain industrial products, such as starch and cassava flour (*tapioca*): peeling and washing, grating, pressing, disintegration, sifting, drying, milling and screening (Tan et al., 2017).

Cassava composition

Tubercle of cassava is organically rich in starch and carbohydrates, also containing small amounts of protein, vitamins and minerals (Lancaster et al., 1982). The protein contents of the *in natura* and dry cassava are 1 and 1.41%, respectively (Table 1). Soccol (1996) reported that *in natura* cassava tubers has moisture 65%; 0.9% of ash and 0.03% of phosphorus (P).

The main composition of cassava is starch and carbohydrates, proteins, vitamins and minerals trace (Lancaster et al., 1988). The carbohydrate contents of the *in natura* cassava are estimated at 35% (Kuiper et al., 2007). Montagnac et al. (2009) assumed the carbohydrate content of the whole cassava root, and peeled roots as 37.9, 31 and 28.8%, respectively. It also contains significant amounts of calcium (Ca), phosphorus (P), zinc (Zn), magnesium (Mg), copper (Cu), iron (Fe), manganese (Mn), potassium (K) and vitamins, such as vitamins C, folates, thiamine, pyridoxine (B6 vitamin), ribofin and pantethenic acid. Another important feature of this biomass is their high oxygen content, which can be higher than 35%, approximately ten times higher than in high-grade coal, which is below 4% (Demirel, 2014).

Torquato et al. (2017) used thermogravimetric analysis (TG) to perform the proximate analysis for several biomass samples. This method describes the determination of moisture, fixed carbon, volatile materials and ash. Veiga et al. (2016) also used thermogravimetric analysis for samples from three cassava plant parts, that is seed stem, coarse and fine stems.

Veiga et al. (2016) presented results of elemental analysis for the different crops and cassava varieties and observed that few variations were found between the different plant parts, except for nitrogen (N), which presented highest amounts, that is 1.7% for thin stems and 0.27% for thick stems. In other cassava varieties (IAC 14 and IAC 90) the N concentration ranged between 0.55 and 0.80%, respectively. It was observed that knowledge of the N content is necessary for estimating the nitrogen oxide (NO) formation through the NO-fuel mechanism in wastes combustion processes (Pattyia et al., 2011).

Veiga et al. (2016) presented in their study the amount of cellulose, hemicellulose and lignin of parts from cassava harvest residues, as shown in Table 2. It was

Table 1. Physical-chemical properties of cassava tubers (100 g).

Composition	Units	Fresh weight	Dry weight	References
Calories	cal	135	335	Okudoh et al. (2014)
Peel	%	10 – 20	n. a.	Lancaster et al. (1982)
Cork layer	%	0.5 – 2.0	n. a.	Kuiper et al. (2007)
Edible portion	%	80 – 90	n. a.	Soccol (1996)
Moisture	%	62 – 66	15 - 19	Lancaster et al. (1982)
Total solids (TS)	%	38	81	Lancaster et al. (1982)
Volatile solids (VS)	%	99	98	Lancaster et al. (1982)
Protein	g	1	1	Lancaster et al. (1982)
Total nitrogen	%	0.22	0.46	Lancaster et al. (1982)
Lipid	g	0.20	0.50	Lancaster et al. (1982)
Starch	g	18 – 32	81	Lancaster et al. (1982)
Fibre	g	1.10	1.20	Lancaster et al. (1982)
Carbohydrate	%	35	n. a.	Kuiper et al. (2007)
Total carbon (TC)	%	19	40	Soccol (1996)
Ash	g	0.9 – 1	2	Lancaster et al. (1982)
Calcium	mg	26	96	Lancaster et al. (1982)
Phosphorus	mg	32	81	Lancaster et al. (1982)
Iron	mg	1	8	Lancaster et al. (1982)
Sodium	mg	2	n. a.	Lancaster et al. (1982)
Potassium	mg	394	n. a.	Lancaster et al. (1982)
B2 Vitamin	mg	0.04	0.06	USDA (2003)
C Vitamin	mg	34	0.00	USDA (2003)
Niacin	mg	0.60	0.80	Lancaster et al. (1982)
Cyanide	%	n. a.	2	Lancaster et al. (1982)

n. a. not available.

Table 2. Amount of cellulose, hemicellulose and lignin of parts from cassava residues (Veiga et al., 2016).

Cassava parts	Cellulose	Hemicellulose	Lignin
Seed stem	39.93	11.73	17.87
Thin stalk	37.67	11.77	22.60
Thick stalk	40.73	12.14	20.05

observed that cellulose amount ranged from 37 to 41%; hemicellulose between 11 and 12% and lignin from 17 to 23%, indicating its has lignocellulosic material characteristic.

Table 3 presents the chemical composition of cassava wastes and comparison with other biomasses commonly used for biofuel, such as sugarcane bagasse, rice straw, yard waste, switch grass, wheat straw and eucalyptus. In general, cassava garbage characteristics resemble most other biomasses, considering elemental analysis (Veiga et al., 2016).

Cassava cultivation

Cassava belongs to the family Euphorbiaceae. This crop grows on infertile land with minimal need of chemical products, such as fertilizers, herbicides and insecticides; making it one of the cheapest and most sustainable agro-based feedstocks. Cassava is cultived primarily in tropical climate, with approximately 70% of their production occuring in subtropical and tropical regions. It is mainly cultivated by small-scale farmers in Africa, Latin America and Asia (Zhang et al., 2016). Cassava is replanted, using the cut stem in their harvest. The stems are cut, ranging from 20 to 25 cm long and planted in a slanting or angular position of 45°, burying them in the soil with one-third of their stems above the surface, ensuring that lateral buds point towards the sun direction, ensuring that the same germinates (Edhirej et al., 2017). Conventionally, it is recommended that the stems are

Table 3. Chemical composition of cassava wastes to comparison with other typical biomasses.

Elemental composition% (dry basis)	Cassava waste[1]	Sugarcane bagasse[2]	Rice straw[3]	Yard waste[3]	Switch-grass[3]	Wheat straw[4]	Eucalyptus[4]
C	44.12	40.34	38.24	41.54	46.68	44.92	50.15
H	6.44	5.66	5.20	4.79	5.82	5.46	7.45
O	48.62	47.91	36.26	31.91	37.38	41.77	39.64
N	0.81	0.58	0.87	0.85	0.77	0.44	0.50
S	<0.2	0.17	0.18	0.24	0.19	0.16	0.02
Cl	<0.3	0.26	0.58	0.30	0.19	0.23	0.55

[1]Veiga et al. (2016); [2]Bizzo et al. (2014); [3]Jenkins et al. (1998); [4]Cuiping et al. (2004).

planted at a spacing of 1 × 1 m on the crest of ridges or mounds, which will give a plant population of 10,000 stands ha^{-1} (Agyepong and Barimah, 2017).

MAIN TECHNIQUES OF PRETREATMENTS FOR THE CASSAVA BIOMASS - AN OVERVIEW

Agricultural biomasses (focus of this review paper) present physical-chemical properties that can be considered for thermal engineering applications, such as: density, fluxability, grindability, moisture sorption, ash and volatile materials content, thermal properties and energy content. Therefore, it is necessary for choosing the correctly pretreatment techniques (Cai et al., 2017). Generally, technologies of pretreatment are sudivided into three major groups, that is thermal, chemical and biological. Although each method presents some advantages, one specific method cannot be applied for all biomasses type. Fundamental understanding of various technologies of pretreatment, different biomass composition, the relationship between feedstock composition and pretreatment methods, can match significantly the best pretreatment method or combinations of this for a specific feedstock. Biomass pretreatment for the reduction of their recalcitrance is a necessary step for bioethanol production (Himmel, 2007). Therefore, the main components of the cassava (bark, stem and leaves) need to be pretreated to unlock their cellulose and hemicellulose contents, which compose more than 50% of their dry weight (Aripin et al., 2013; Nanssou et al*.,* 2016).

Mechanical pretreatment

Mechanical pretreatment used in some biomasses is essential to improve particle distribution and densification, enzymatic accessibility and bioconversion affectivity (Peltola et al*.*, 2004). According to Barakat et al. (2014), such pretreatment also increases porosity and bulk density, improves flow properties and generates new surface areas, without the production of toxic side streams. These pretreatments involve the physical dispersion of substrate components, reducing particle size and increasing the available surface area (Liau et al*.*, 2011). For the cassava biomass, this reduction in particles size facilitates a faster moisture adsorption and makes nutrients readily available to the microorganisms that are responsible for anaerobic fermentation and therefore, leads to better methane gas production (Salomoni et al*.*, 2011). The mechanical breakdown that usually occurs in the cassava cell walls can be monitored by increasing the oxygen-soluble chemical (COD) content of the substrate.

The mechanical methods need an initial energy to disrupt noncovalent forces between the cassava cells (Muñoz et al., 2006). Chemical modifications of the organic matrix rarely are observed, and when these occur, they are not significant (Barakat et al., 2014). Peltola et al*.* (2004) observed an increase of approximately 60% in soluble COD content by using mechanical pretreatment for samples of municipal solid wastes (MSW). However, more researches need to be carried out, focusing on the efficiency of this pretreatment for cassava residues, as well as the particle size effect of this biomass for methane production via anaerobic fermentation processes. In addition, this mechanical method has been applied to maintain the integrity of plant enzymes and improve the digestion of energy crops. For cassava biomass, the main problem in the use of this method is the energy required for their milling, which can compensate the gains obtained in biogas production (Buaban et al., 2010). As can be seen, there are some advantages and disadvantages for this pretreatment technique. For example, using an agitated ball mills, a solubilization among 10 and 30% and an increase from 10 to 20% in the biogas production can be obtained (Buaban et al., 2010; Liao et al., 2011). On the other hand, main disadvantages for using this technique are the capital and operational costs (Salomoni et al., 2011).

Chemical pretreatment

Chemical pretreatments such as acid, alkaline or ozone

can be used, which enable a solubilization from 30 to 60% for the insoluble substrates (Silverstein et al., 2007). Ozone treatment produced a 41% increase in biogas production, while alkaline treatment produced 25 to 100% increase in biogas yields, as well as in methane production (Edhirej et al., 2017). The main disadvantage of chemical treatment lies with the cost for acquiring the chemicals. Ozone treatment is highly economical in a commercial scale (Mosier et al., 2005). Zhang et al. (2011) used this method for cassava treatment and reported methane yields of 259.46 ml g^{-1} of volatile materials destroyed.

Hydroxides of sodium (NaOH), potassium (KOH), calcium ($Ca(OH)_2$) and ammonium (NH_4OH) are the alkali pretreatments used more for bioethanol production (Rabelo, 2010; Rezende et al., 2011; Cruz et al., 2017). The $Ca(OH)_2$ used in this process can be recovered using lime kiln technology (Cai et al., 2017). This method is also known for causing chemical swelling in the cellulose fibrous (Mosier et al., 2005; Cruz et al., 2017), in which occurs saponification reactions and salvation, leading to the disruption of the cross-links between hemicellulose and other components; hence, increasing the biomass porosity (Sun and Cheng, 2002; Cruz et al., 2017). More specifically, cross bonds between ester, lignin and xylan are disrupted, producing the delignification process. Comparatively, alkaline pretreatments are performed at lower temperatures, approximately 60°C and do not require complex reactors that are appealing to be employed on farms (McIntosh and Vancov, 2010).

Acid pretreatment, in particular, using sulfuric acid (H_2SO_4) is the most employed chemical pretreatment for lignocellulosic biomass, where polysaccharides (mainly hemicellulose) are hydrolyzed to monosaccharides, leading to higher accessibility of cellulose to enzyme hydrolysis (Rabelo, 2010; Rezende et al., 2011; Cruz et al., 2017). Acid pretreatment can be performed either under low acid concentration and high temperature or under higher acid concentration and lower temperature (Taherzadeh and Karimi, 2008). According to Xu et al. (2007), soybean straw samples were soaked in ammonia liquor (10% NH_4OH) for 24 h at room temperature, and it was observed that their hemicellulose and lignin contents decreased by 41.45 and 30.16%, respectively. Generally, to use concentrated acid is more economical, when the process is performed at low temperatures (Girio et al., 2010; Mood et al., 2013).

Zhang et al. (2011) investigated cassava residues pretreatment by thermally diluted sulfuric acid hydrolysis by means of statistically designed experiments. Results obtained indicated that the hydrolysis, using dilute sulfuric acid, is adequate to predict the ideal pretreatment condition, which showed to be effective for cassava wastes pretreatment, increasing the methane yield. Martín et al. (2017) carried out the chemical characterization of cassava stems from different origins (South China 205-SC205, Xinxuan 048-XX048 and South China 5-SC5), where cassava stems were submitted for saccharification, including starch hydrolysis, pretreatment with sulfuric acid or 1-ethyl-3-methylimidazolium acetate and enzymatic hydrolysis of cellulose. Pretreatment with OAc resulted in 20% higher glucan conversion than pretreatment with acid.

The use of these chemical pretreatments also presents some advantages and disadvantages. For instance, with the alkaline method solubilization ranging from 30 to 60% can be obtained (Taherzadeh and Karimi, 2008), although the increase of non-biodegradability materials is the main disadvantage for using this technique. Ozone technology can improve the solubilization at 30%, but this pretreatment cause destruction of cell structure (Mshandete et al., 2008).

Biochemical pretreatment

Among pretreatments different for the biomasses discussed in this review paper, biochemical pretreatments present advantages, such as simplicity in experimental operation and low capital investment, which makes them more attractive (Mshandete et al., 2005; Chen et al., 2010). Biochemical pretreatments commonly use microorganisms, such as brown, white and soft rot fungi for lignin degradation and hemicellulose from lignocellulosic biomasses (Sindhu et al., 2016). Second, Sindhu et al. (2016) biochemical pretreatments, using white rot fungi that are able to degrade lignin, seems very promising, because less energy is consumed and the environment is not damaged. Currently, studies are being performed for detecting alterations in the structure, chemistry composition and enzymatic hydrolysis of lignocellulosic biomass after biological pretreatment (Chen et al., 2010). Shen et al. (2015) reviewed two hybrid processes, including the characteristics of fermentative substrates produced in the thermochemical stage and microbial utilization of these compounds in the fermentation stage.

Zang et al. (2011) analyzed the cassava residues pretreatment with distillery wastewater mixture by anaerobic digestion, using a microbial consortium as inoculations in batch bioreactors at 55°C. Results showed that maximum methane yield of 259.46 ml g^{-1} volatiles materials for cassava residues was obtained for 12 h pretreatment by a microbial consortium, which was 96.63% higher than control, i.e. 131.95 ml g^{-1} volatiles materials.

Biogas production

Biogas is produced from anaerobic digestion (AD) of organic materials by microbes. AD is a microbial decomposition process of organic materials in oxygen

absence for biogas production. AD occurs in four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Wang et al., 2012). Biogas is constituted mainly by methane, carbon dioxide and hydrogen sulphide traces, ammonia, hydrogen and nitrogen. Methane is the component that provides the high energy value (Balat and Balat, 2009). High heating value (HHV) of biogas ranges from 5000 to 7000 kcal m^{-3} (depends on methane content). In practice, different substrates spectrums are combined into AD process. This AD process is called anaerobic co-digestion (ACD) process. ACD processes are known for biogas synergistic yield, because combination yield is higher than sum of AD individual yields (Khalid et al*.,* 2011). Adelekan and Bamgboye (2009) studied the ACD productivity of several mixture ratios of cassava peels with livestock wastes (poultry, piggery and cattle) and concluded that for each case, ACD produced improvement in the biogas yield. Also it was observed that each mixture provided the best yield in the 1:1 constituents ratio by mass. For any ratio, ACD with piggery wastes presented the best yield, followed by cattle and poultry wastes.

Thermal pretreatment

According to Ferrer et al. (2004), thermal pretreatment can increase the biogas production and methane yield of certain substrates, but is not an effective technique in all cases. For example, thermal pretreatment of hyacinth water at 80°C increased slightly the solubility, with few or no effect on the anaerobic digestion (Ferrer et al., 2004). Pasteurization process of abattoir wastes at 70°C for 1 h, produced a four-fold increase in methane yields, but the application of this pretreatment cannot be generalized for the different biomasses (Faloye and Kana, 2017). For Chandra et al. (2012), temperatures below 100°C are used to breakdown plant cells, increase membrane fluidity and hydrolyze polymers, resulting in a soluble COD release of approximately 35%. This thermal method causes modifications in the chemical equilibrium of the exopolymers in the lignocellulosic biomasses.

Thermal pretreatment was applied by Aruwajoye et al. (2017) for the optimal release of fermentable sugar from cassava husks. The authors used the response surface design method to investigate the effect of immersion temperature, immersion duration and autoclave, acid concentration and solid loading on the sugar yield reduction, obtainig optimal pretreatment conditions and immersion temperature of 69.62°C. Thermal pretreatment may be used for cassava residues, but their cost must be weighed against the benefits derived from increased biogas production rates (Norberg, 2004).

Ultrasonic pretreatment

Pretreatments based on ultrasound irradiation has been employed as isolated technique or combined with other technologies. Such combinations include acid pretreatments, alkaline, ionic liquid and ozone or with a physical technique, for example, microwave irradiation, thermal and supercritical carbon dioxide, for the pretreatment of lignocellulosic biomasses and wastes for improving biofuel production (Saifuddin and Fazlili, 2009; Erden and Filibeli, 2010; Tian et al., 2016). Ultrasound present a spectrum ranging between 20 and 10 MHz, and their tone is above human hearing, which can detect sounds up to 16 kHz (Mood et al., 2013). This process catalyzes the depolymerization of biopolymers, emulsification, and extraction of tanning vegetables oils from almond, ginger, and wood seeds (Bundhoo and Mohee, 2017).

Tian et al. (2016) reported that some applications of the irradiation processes via ultrasound can be implemented on municipal wastewater pretreatment to disrupt flocks, production of biodiesel from microalgae and bioethanol from cassava chips. Laboratory scale studies using ultrasonic pretreatment showed that solubilisation degree was between 30 and 90% and increase in the biogas production from 5 to 70% (Bundhoo and Mohee, 2017). Lehne et al. (2000) reported that use of this technique promote a reduction in the average particle size, increasing the disintegration degree of the sewage sludge samples; however this cannot be suitable for energy crops such as cassava.

Vera et al. (2004) used an ultrasound 20 kHz and the power supply of 500 watts to disintegrate sewage sludge and, consequently, to increase the fermentation rates, but cannot be suitable for lignocellulosic biomass. For the cassava biomass, the use of ultrasound pretreatment cannot be ideal due to the requirement of a high energy for disintegrating of the cell walls (Clarke, 1999; Saifuddin and Fazlili, 2009).

Wet explosion pretreatment

The wet explosion process was developed as a combination of thermal and chemical oxidation to treat biomasses with high concentrations of sugars. However, biogas production was not significantly increased by this technique (Chandra et al., 2012). Wang et al. (2012) used wet explosion pretreatment for enhancing methane production from energy crops, such as cassava and other agricultural residues. The results showed an increase in the sugars release after pretreatment, but not implying at higher methane yield (Wei et al., 2015).

Steam explosion pretreatment

Steam explosion pretreatment is an extensively investigated thermomechanical and chemical method, involving the structural components breakdown of lignocellulosic materials by steam-heating and shearing

Table 4. Effects of different pretreatments on the chemical composition and structural of lignocellulosic biomasses and their limitations (Mood et al., 2013).

Pretreatment method	Increase specific surface	Hemicellulose removal and solubilization	Lignin removal	Inhibitor compounds formation	Drawback and disadvantages
Physical	++	–	–	–	High energy consumption
Acid	++	++	+	++	Equipment corrosion, degrading produce sugar
Alkaline	++	+	++	+/–	Neutralization of pretreated slurry
Ionic liquid	++	+	+	–	High cost of ionic liquid
Organosolv	++	++	++	–	Recovery and recycle of solvent by evaporation, high cost
Steam explosion	++	++	+/–	++	Incomplete disruption of lignin–carbohydrate matrix,
CO_2 explosion	++	++	–	–	High pressure requirement, does not affect on lignin and hemicelloluse
Biological	++	+/–	++	–	Low hydrolysis rate, large space requirement, watchful control condition of microorganism growth

mechanical, that is due to sudden decompression, moisture evaporation and auto-hydrolysis of glycosidic bonds (Mood et al., 2013; Cai et al., 2017). In this process, biomass particles are heated using pressurized steam, with pressure between 20 and 50 bar, and temperature ranging from 160 to 270°C, during few minutes. After this step, the pressure is released to atmospheric pressure, condensed moisture evaporates and lignocellulosic matrix desegregation takes place (Mabee et al., 2006; Mood et al., 2013). Okudoh et al. (2014) related that this pretreatment causes hemicellulose hydrolysis, lignin transformation due to high temperature and increases cellulose crystallinity, promoting crystallization of the amorphous portions.

Comparison between the effects of different pretreatments on the chemical composition and structure of lignocellulosic biomasses and their possible limitations are presented in Table 4.

THERMOCHEMICAL CONVERSION PROCESSES

Current technologies available for converting biomass into fuels can be classified into four categories based on their methodologies: biochemical, chemical, thermal, and thermochemical conversion. Thermochemical processes are commonly employed for converting biomass into biofuels with high heating value (Phillips et al., 1990; Ferreira et al., 2017). Biomass thermochemical conversion includes a great number of processes, such as direct combustion, liquefaction, gasification, pyrolysis and oxy-fuel combustion (Bridgewater, 2001; Park et al., 2012). From techniques presented, pyrolysis is the more usual of the biomass thermochemical conversion processes to produce solid and liquid fuels, both are easy to handle and transport (Van de Velden et al., 2010). Figure 2 shows the possibilities of converting the stored energy within biomass directly into heat via combustion/co-firing or transformed into solid fuels (charcoal), liquid (bio-oils) or gaseous (synthetic gas) with various utilization purposes (Bridgewater, 2001).

One of the disadvantages of using biomass as fuel in thermochemical processes is their high moisture content (Phillips et al., 1990). Although the combustion reactions are exothermic processes, the water evaporation is endothermic (Park et al., 2012). For maintaining the self-sustaining combustion process, moisture content of biomass fuels cannot exceed 65% (Science Daily, 2010). Even with moisture content within the acceptable maximum limit, the fuel high heating value (HHV) is negatively correlated with the amount of water (Quaak et al., 1999).

Figure 3 shows the negative linear relationship between the moisture content and HHV. As the moisture content increases, both the HHV and Low Heating Value (LHV) decrease (Phillips et al., 1990). HHV and LHV are used to describe the heat production of a unit fuel amount during their complete combustion. In determining the HHV and LHV of a fuel, liquid and vapor phases from water are selected as the reference states, respectively (Goyal et al., 2008). As HHV incorporates the heat condensation of water vapor during combustion, it is noted that the HHV curve is always above LHV (Quaak et al., 1999).

Direct combustion

Many studies have been devoted to agricultural

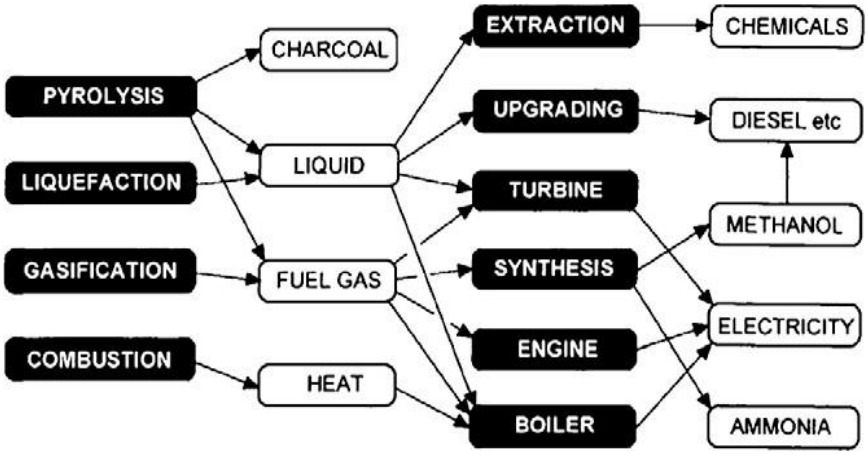

Figure 2. Thermochemical processes for bioenergy production and their corresponding products.
Source: Bridgewater (2001).

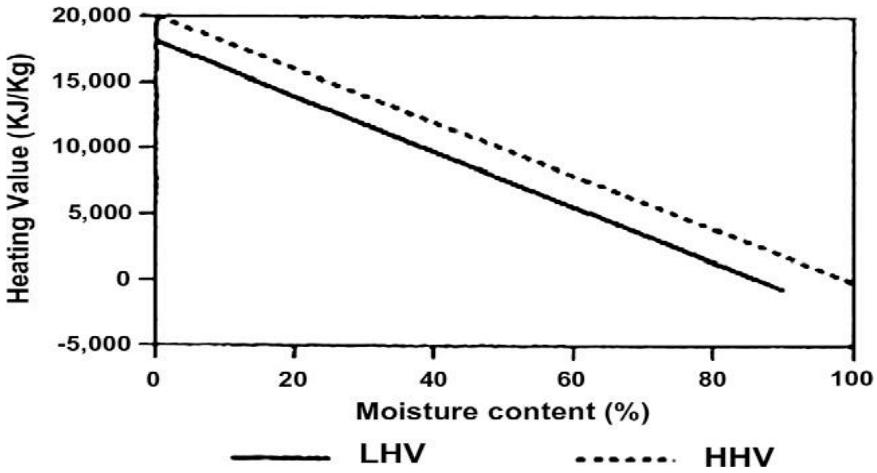

Figure 3. Relationship between heating value and moisture content of biomass fuel (Quaak et al., 1999).

wastes combustion in fluidized bed systems with sand (Kaynak et al*.,* 2005; Madhiyanon et al*.,* 2010; Pécora et al., 2014). These studies focused mainly on fluorinated gas emissions, efficiency and ash related problems, such as fouling and bed agglomeration (Zhang et al*.,* 2011). Combustion performance in terms of efficiency and emissions has been reported to depend heavily on fuel properties as well as on system design characteristics and operating parameters, such as fluid velocity, bed temperature, fuel feed rate, etc (Isahak et al., 2012; Karan et al., 2011).

Combustion is a process widely used to convert stored chemical energy into biomass then into heat, mechanical energy or electricity, using various processes and equipments, such as stoves, ovens, boilers, steam turbines, turbo generators, etc (Demirbas, 2000; IEA, 2006). This is a known technology, although improvements in performance are still possible (Quaak et al*.,* 1999). Biomass combustion produces hot gases at temperatures between 800 and 1000°C and it is possible to burn any biomass type. However, this process is more reliable for application with moisture content below 50% (McKendry, 2002).

Fixed or fluidized beds and drag reactors are three typical combustion systems, with an increase in the velocity of the carrier gas inside the reactor (Laursen and Grace, 2002). A higher gas velocity implies an intensive mixing of the feedstock, which improves combustion efficiency and heat exchange rate (Madhiyanon et al., 2010). The flushed flow systems are expected to exhibit the best performance among these three types of combustion systems (Pattiya et al., 2012). However, no

study about direct combustion of cassava residues was found.

Pyrolysis

Pyrolysis is a thermal decomposition process that takes place in the absence of oxygen (inert atmosphere) to convert biomass into solid charcoal, liquid (bio-oil) and gases at elevated temperatures. Pyrolysis is considered an industrially realized process for biomass conversion (Truman et al., 2004; IITA, 2005). This process can be divided into three subclasses, that is slow, flash and fast pyrolysis (Karan et al., 2011; Pattiya et al., 2012). According Zabaniotou and Ioannidou (2008) the slow pyrolysis occurs under a low heating rate, which obtain more charcoal yield, while the flash pyrolysis is a rapid heating rate process occurring at moderate temperatures (400 to 600°C), obtaining maximized volatile products at short residence time and occurs at high temperature and longer residence times, increasing the biomass conversion and returning more gas product.

For Weerachanchai et al. (2011), the first stage, also called pre-pyrolysis, occurs between 120 and 200°C with a slight weight loss, where some internal rearrangements, as bond breakage, free-radicals appearance, and formation of carbonyl groups are observed, with a release of small amounts of water (H_2O), carbon monoxide (CO), and carbon dioxide (CO_2). The second-stage is itself a pyrolysis process, during which solid decomposition occurs, accompanied by a significant weight loss from the initially fed biomass (Jiménez and Ballester, 2006). Finally, in the last stage occurs the continuous char devolatilization caused by the further cleavage of C–H and C–O bonds (Maschio et al., 1992).

Karan et al. (2011) investigated the pyrolysis process of cassava rhizome, utilizing flue gas in the lab-scale metal kiln. It was reported that the charcoal yield for the dry cassava rhizome ranged from 26 to 35%, depending on the pyrolysis temperature and the fast pyrolysis time was found from 19 to 38 min. In particular, fast pyrolysis favours the formation of liquid products, but inhibits solid chars formation (Maschio et al., 1992). Their liquid products (bio-oils) are composed of an aqueous phase, which contains several organo-oxygen compounds of low molecular weight, and a non-aqueous phase (tar), which includes a variety of insoluble aromatic organic compounds of high molecular weight (Yanik et al., 2009; Zhang et al., 2011).

Some studies on the utilization of cassava residues for the bio-oil production by pyrolysis process have been reported (Pattiya, 2011; Pattiya et al., 2012). Pattiya and co-workers used rhizome and stalk of cassava as feedstocks to obtain bio-oil by fast pyrolysis process in two reactors: fluidized bed reactor (Pattiya, 2011) and free fall reactor (Pattiya et al., 2012). Weerachanchai et al. (2011) carried out slow pyrolysis from cassava pulp residues and palm kernel cake and their formed products, included solids, liquids and gases.

Pattiya et al. (2012) used the fast pyrolysis of agricultural residues, i.e. cassava plantations, in free fall reactor of laboratory scale to investigate effects of this biomass and the pyrolysis conditions, such as reactor temperatures, condensation, nitrogen flow rate and execution duration in the distribution from pyrolysis products. For maximizing the bio-oil yield, optimum reactor temperatures were reached between 350 and 450°C. It was observed that for the reactor temperature of 450°C and condensation primary temperature of 10°C, about 70% weight bio-oil yield for the cassava stem pyrolysis was obtained. It was also verified that the minimum nitrogen flow rate for obtaining high bio-oil content was 1.5 L min^{-1}.

Suttibak et al. (2012) reported experimental proceeding of rapid pyrolysis from cassava rhizome in a fluidized bed reactor incorporated with a hot steam filter. Results showed that ideal pyrolysis temperature was around 472°C, which produced a maximum bio-oil yield of 63.23% on a dry basis.

Gasification

According to Couto et al. (2013), gasification is the carbon based solid material conversion into gaseous fuels at high temperatures, usually from 800 to 900°C, in order to optimize gas production. Gas produced with a LHV ranging from 4 to 6 MJ Nm^{-3} can be directly burned or used as fuel for engines and gas turbines (McKendry, 2002). Badin and Kirschner (1998) found that high efficiencies, approximately 50% are achievable using combined cycle gas turbine systems, where the residual gases of the turbine are recovered to produce superheated steam for using into a steam turbine. Most commercial gasifiers are downdraft type, fluidized bed systems and upstream type, such classification depends on the biomass feed-way, which can be from top, bottom or side of the gasifier (Rezaiyan and Chereminoff, 2005). Another important aspect is the bed type, for example ice beds or fixed. One reactor specific type is not necessarily suitable for the full power ranges, for example each reactor is operated in an adequate range. For example, fixed bed (upflow and downdraft) is suitable for smaller scales, which ranges from 10 to 10 MW; fluidized bed is more suitable for intermediate units from 5 to 100 MW; while trailed bed reactors are used for large scale power plants higher than 50 MW (Basu, 2010).

A detailed comparison between biomass gasification and combustion was provided by Rezaiyan and Chereminoff (2005) and is summarized in Table 5. Generally, combustion aims on heat generation, whereas the gasification creates valuable gaseous products that can be used directly for combustion or stored for other

Table 5. Comparison between gasification and combustion processes (Rezaiyan and Chereminoff, 2005).

Features	Gasification	Combustion
Purpose	Creation of valuable, environmental friendly,usable products from waste or lower value material	Generation of heat or destruction of waste
Process type	Thermal and chemical conversion using or no limited oxygen	Complete combustion using excess oxygen (air)
Pressure	Atmospheric to high	Atmospheric
Raw gas composition (before gas cleanup)	H_2, CO, H_2S, NH_3, and particulated materials	CO_2, H_2O, SO_2, NO_x, and particulate materials
Solid byproducts/products	Char or slag	Bottom and fly ashes

applications (Mok et al., 1992). Fixed bed generally produces low heat synergies and is suitable for small or medium scales in thermal applications (Pattyia, 2011). Since there is no mixing within the reactor, reaction uniform temperatures are difficult to be achieved (Moster et al., 2005). Fixed bed fans include upstream (countercurrent), downdraft (concurrent), crossflux and gas open (Araque et al., 2008).

Oxy-fuel combustion

Peng et al. (2016) discovered that the CO_2 emissions can be reduced by different ways, i.e., water absorption at high pressure, combustion in the presence of calcium oxide, oxy-fuel combustion, and electrical absorption. Among the CO_2 reduction methods cited, oxy-fuel combustion is considered as one of the most important and promissing options for CO_2 sequestration, due to their ability for a significant reduction in the operating costs (Taniguchi et al., 2002). It was known that oxy-fuel combustion of pulverized biomass in O_2/CO_2 atmosphere can result in an increasing of the char conversion amount and combustion efficiency (Peng et al., 2016; Cruz and Crnkovic, 2016). Taniguchi et al. (2002) reported that NO_x emissions under O_2/CO_2 atmosphere are about 25% those emitted in air atmosphere. In addition, coal oxy-fuel combustion makes it possible to capture and sequester carbon, using technology already available in conventional pulverized coal boilers, and to capitalize on the enormous quantities of money invested in the existing boilers (Riaza et al., 2012). Some researchers (Sengupta and Basu, 1991; Stenseng et al., 1995; Iavarone et al., 2017; Gaikwad et al., 2017) focused their attentions on the development of mathematical models to predict coal combustion, gaseous emissions and combustion chambers performance. It was observed that biomass oxygen emissions are not extensively investigated and applications of oxy-fuel combustion using cassava residues were not found.

FINAL CONSIDERATIONS

After extended research about the several known ways for biochemical and thermoconversion from cassava harvest residues and agricultural residues in bioenergy or biofuels, it was observed that many information and specific applications for this biomass are still lacking, which leads the researchers to the developing of studies that are more applicable to the real situations in each Country. In Brazil, for example, cassava harvest wastes present a great potential for use as bioenergy alternative source, but some care should be taken when these are used as biofuel, due to the high occurrence of ashes in this biomasses, which can cause incrustation in thermal systems. Furthermore, because of the high moisture content presented at the time of harvest, a drying process should be provided before the wastes can be used as biofuel.

Finally, it is understood that correct and adequate use of cassava harvest residues in a sustainable and environmental friendly way, is an important factor for a socio-environmental conscience more concerned with the future of the Planet.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Effect of *Moringa oleifera* leaves extract on the oxidative stress and gastric mucosal ulcer induced by indomethacin in rats

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Indomethacin is commonly used as an anti-inflammatory and pain relieving medication; however, it has the side effect of gastric ulcer formation which is an actual common gastrointestinal illness that may result in dangerous complications and even death. Various diseases have been treated widely by the use of oriental herbal medicines, this study aims to evaluate the antiulcerative and antioxidative effect of two doses (100 and 50 of *Moringa oleifera* ethanolic leaf extract; MOELE) on indomethacin plus ethanol-induced oxidative gastric mucosal injury in rats. Sixty adult males Wistar rats weighing 170 to 200 g, were divided into equal six groups. First group of rats were administered saline as a vehicle, second group of rats were given indomethacin (15 mg/kg), third and fourth groups of rats were giving MOELE 100 and 500 mg, fifth group of rats were given indomethacin+ MOELE 100 mg, and sixth group of rats were administered with indomethacin + MOELE (500 mg). To study the effect of MOELE on oxidative gastric mucosal injury in rats, two doses were administered 2 h before ulcer induction by indomethacin plus ethanol. The administration continued for two weeks. All rats were sacrificed 24 h after the last dose. Indomethacin group showed significant increases in lesion index (LI) and increase in malondialdehyde (MDA) level, while there was a decrease in superoxide dismutase (SOD), glutathione-S-transferase (GST) and glutathione peroxidase (GPx) activities when compared with the control group (G1). MOELE with two doses mentioned before (groups 5 and 6) were effective to reduce stomach LI and oxidative stress markers (MDA) while increasing significantly the antioxidant biomarkers (SOD, GST, and GPx) compared with indomethacin group (G2). A highly significant decrease in MDA accompanied by a marked increase in SOD, GST, and GPx were recorded in group 6. The results concluded that MOELE has an effective antiulcer and antioxidant activities. It can scavenge the free radicals and protect gastric against ulceration. Also, MOELE could ameliorate the ulcerative side effect of indomethacin.

Key words: *Moringa oleifera,* antioxidant enzymes, indomethacin, lesion index, lipid peroxidation.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) as indomethacin are the most prescribed group of drugs in the world. They are used primarily for pain relief in chronic inflammatory joint disease. They are the main cause of peptic ulceration and its use has been associated with the development of gastrointestinal (GI) symptoms ranging from simple dyspepsia to life-threatening GI bleeds and perforations (Yap et al., 2015).

Ulcer development destroys the mucosal barrier exposing the underlying stomach tissue to the destructive action of acid and pepsin (Vander, 1998). Numerous factors have been implicated in the pathogenesis of peptic ulcer disease, which may be acquired during life, although some of these may have already been determined (Ghasi, 2000).

Gastric hyperacidity and ulcer are very common, causing tremendous human suffering nowadays. It is an imbalance between damaging factors, within the lumen and protective mechanisms within the gastro duodenal mucosa. Although prolonged anxiety, emotional stress, hemorrhagic surgical shock, burns, and trauma are known to cause severe gastric irritation, the mechanism, however, is still very poorly understood (Rao et al., 2000). The problems of ulcer due to indomethacin could be prevented by herbal treatment. One of these promising medicinal therapy is *Moringa oleifera*. The advantage of choosing a medicinal plant includes its easy availability, low cost and nearly no side effect compared with the synthetic medications.

M. oleifera Lam (syn. *M. pterygosperma*; commonly known as "The Miracle Tree," as almost every part of it is useful for humans)." It has medicinal and nutritional value; it is also widely distributed throughout the world in Himalayan tracts, India, Pakistan, and Africa. It could be found even in the harshest and driest of soils (Luqman et al., 2012). Moringa plants are used as a food source with valuable properties in humans. Genus moringa contains vitamin C, vitamin A, potassium, iron, calcium and the protein quality of moringa leaves is claimed to be similar to eggs and milk (Fahey, 2005).

There are 36 anti-inflammatory compounds (phenolic derivatives and isothiocyanate) and 46 antioxidants (carotenoids, ascorbic acid, phenolic compounds, and flavonoids). These compounds naturally occur in the moringa plant (Anwar et al., 2007; Goyal et al., 2007; Adedapo et al., 2009). The leaves are reported to have anti-inflammatory, diuretic, antispasmodic, and hypotensive activity (Fayazuddin et al., 2013). The antioxidant property of moringa may be due to the presence of phenolic compounds (Bharali et al., 2003).

The existence of reactive oxygen species (ROS) leads to oxidative stress. It causes disturbances in the cellular metabolism (Breitenbach and Eckl, 2015). Oxygen free radicals mediate tissue injury and destroy the integrity of biological tissues. Also, it is associated with lipid peroxidation, which causes tissue damage by destroying cell membranes and releasing some of their intracellular components. ROS also can cause mucosal damage through the retrogression of the epithelial basement membrane components. Indeed, biological system's ability to repair oxidative damage or to neutralize the reactive intermediates including peroxides and free radicals (Demir et al., 2003; Suzuki et al., 2012).

The gastric mucosa plays an important role in the physiological function of the stomach. This mucosa acts as a gastric barrier, which protects deeper located cells against the detrimental action of the gastric secretory components. The pathogenesis of gastric mucosal damage includes ROS that cause tissue damage, mainly due to increased lipid peroxidation (Kwiecien et al., 2014).

Antioxidant, anti-inflammatory, and immunomodulatory properties of biophenols are abundant in *M. oleifera* Lam. suggesting that they may have beneficial effects on inflammatory bowel diseases like gastric ulcers (Mahajan et al., 2007; Shaila et al., 2010).

Recently, Omodanisi et al. (2017) reported that *M. oleifera* has effective phytochemical ingredients that offer protection action against diabetic-induced renal damage, ROS and inflammation and could, therefore, show a role in decreasing diabetic problems, mainly in developing nations such as Africa where the majority cannot afford to purchase medicines.

The present study aims to use inexpensive, effective and readily accessible medication with a low side effect for the ulcer therapy. Therefore, the current study was carried out to assess the antioxidant activity of *M. oleifera* ethanolic leaf extract (MOELE) using *in vivo* acute models of ulcer that cause oxidative gastric damage in rats. Also, this study aims to scientifically confirm the use of *M. oleifera* leaves in the treatment of gastric ulcer.

MATERIALS AND METHODS

Chemicals

Indomethacin was purchased from Chiesi Pharmaceutics SPP, Parma, Italy. Ethanol and kits used for measurement of malondialdehyde (MDA), glutathione-S-transferase (GST), superoxide dismutase (SOD), and glutathione peroxidase (GPx) were purchased from Diamond and Sigma Company.

Plant material extraction

Air-dried powder (200 g) of *M. oliefera* leaves were soaked in 70% ethanol for 2 days and filtered. The filtrate was distilled using a rotary evaporator until dryness. The remaining solid residue was dissolved in distilled water, filtered and the filtrate was evaporated until dryness (dry mass 10 g). The dried mass was diluted with normal saline (100 mg/ml) and used in the experiments.

Animals

This study includes sixty adult males Wistar rats weighing 170 to 200 g. Wire bottomed cages were used for housing of the animals

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under controlled conditions of temperature (20 to 24°C), humidity and12/12 h light/dark periods. Rats were fed with chow pellets and tap water was freely accessible. Animals were prevented from food overnight before the experiment. The animal experiments were approved by the Committee of Scientific Ethics at University of Dammam and consistent with its guidelines (IRB-2016-10-155).

Animals were randomly divided into six groups (10 rats each) as follows: Control group, received vehicle (0.5 ml vehicle) for two weeks; Group 2 (Indomethacin), rats were given oral administration of indomethacin at a concentration of 15 mg/kg-body weight/0.5 ml water in addition to 0.5 ml absolute ethanol for induction of gastric mucosal haemorrhagic injury (De La Lastra et al., 1999); Group 3 (100-MOELE), rats were given a single dose of MOELE, 100 mg/kg-body weight orally three times/week for 2 weeks; Group 4 (500-MOELE), rats received a single dose of *M. oliefera* extract, 500 mg/kg-body weight, orally three times/week for a period of 2 weeks; Group 5 (100-MOELE+Indomethacin), rats received a single dose of MOELE, 100 mg/kg-body weight, 2 h prior to induction of gastric mucosal haemorrhagic injury as in group 2; Group 6 (500-MOELE+Indomethacin), rats received a single dose of MOELE, 500 mg/kg-body weight, 2 h prior to induction of gastric mucosal haemorrhagic injury as in group 2.

Rats were sacrificed under ether anaesthesia at the end of the experimental period (the day after receiving the last dose). Abdomens were opened and stomachs were exposed. Then stomachs were opened along the greater curvature. The tissue of the stomach was washed using normal saline. Examination of tissue and mucosal injury can be carried out microscopically using a light microscope (Morini and Grandi, 2010). Scores/ratings as described by Okabe et al. (1970), was used to determine the ulcer index as a marker for the severity of gastric lesions a scoring system based on the length and number of hemorrhagic mucosal erosions, a lesion index (LI) of gross mucosal injury was performed as follows: stomach was dissected out, inflated with 12 ml of 2% formalin, placed in 2% formalin to fix both the inner and outer layers, and then opened along the mesenteric attachment or along the greater curvature. The incidence of animals with lesions was noted, and the damaged area (in square millimetres) was measured under a dissecting microscope with a square grid. The sum of the area of all lesions in gastric for each animal was calculated and served as the lesion index. Ulcer index = 10/*X,* where *X* = total mucosal area/total ulcerated area.

Gastric mucosa was removed using a skin-scraping spoon and then homogenized for biochemical assay.

Biochemical analysis

Gastric mucosal preparations were used for measuring the lipid peroxidation product as MDA according to Draper and Hadley (1990). Activity of GST was assayed using the method of Habig et al. (1974), SOD was assayed by Giannopolitis and Ries (1977), and GPx was assayed by Rotruck et al. (1973). The enzyme activity was expressed as unit/mg of protein.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer methods for *post-hoc* analysis. A value of $P<0.05$ was considered statistically significant. Statistical analyses were carried with the aid of a digital computer, using STAT and SPSS version 16.0 programs. Data were presented as mean ± standard deviation (SD).

RESULTS

Generally, an analysis of the results compared the control group with indomethacin to examine its effect as model for the LI and oxidative stress, while groups 3 and 4 were used to monitor if the MOELE alone has a role or not. So compared with (-ve) control groups, no changes were observed due to the administration of MOELE alone. Meanwhile, the effect of treatment MOELE + indomethacin tested in comparison with indomethacin as a control +ve group. Observations revealed that there were no changes in the control group which suggests that handling and surgical procedures had no interference with experimental results.

Administration of indomethacin induced increased in LI (Table 1), elevation in MDA while decreasing the activities of antioxidant markers of GST, SOD, and GPx, indicating rises in the oxidative stress compared with control group (Table 2).

Oral administration of MOELE prior to administration of indomethacin plus ethanol highly significantly reduced the lesion index ($P<0.001$) compared with indomethacin group (Table 1). In addition, the lesion index was significantly reduced with the high doses of MOELE (500 mg/kg) (Table 1).

As shown in Table 1, gastric hemorrhagic lesions had improved in the groups 5 and 6 which received MOELE for 2 h before oral administration of indomethacin and ethanol. These lesions were accompanied by a highly significant rise in the lipid peroxidation level that expressed a high MDA level ($P<0.001$) and highly significant decreases in antioxidant enzymes ($P<0.001$) in group 2 (indomethacin group) when compared with the control (Table 2). While, oral administration of MOELE prior to administration of indomethacin plus ethanol significantly reduced the lesion index of groups 5 and 6 ($P<0.001$) (Table 1), as they might have significantly decreased the rise in MDA concentration and restored the activities of the antioxidant enzymes of GST, SOD and GPx in gastric mucosa when compared with indomethacin-treated rats (group 2) (Table 2). In addition, the administration of *M. oliefera* leaf extract three times/week for 2 weeks (group 6) has more pronounced effect. Moreover, the levels of antioxidant enzymes were not changed in rats of groups 4 and 3 when compared with control due to the administration of MOELE with different concentrations (Table 2).

DISCUSSION

The peptic ulcer is one of the major gastrointestinal disorders; the treatment of peptic ulcer is directed against either reduction of the aggressive factors or enhancement of defensive mechanism. A number of drugs, including proton pump inhibitors and H_2 receptor antagonists, are available for the treatment of peptic ulcer, but the clinical evaluation of these drugs has shown the incidence of relapse, side effects and drug interactions (Anoop and Jagadeesan, 2003).

Table 1. Effects of MOELE on macroscopic ulcer (Lesion index) in various groups in comparison with indomethacin-treated and control group.

Group	LI*
Group 1 (Control)	-
Group 2 (Indomethacin)	42.8+4.6[a]*
Group 3 (100mg-MOELE)	-
Group 4 (500mg-MOELE)	-
Group 5 (100mg-MOELE+Indomethacin)	19.3+2.47[b]
Group 6 (500mg-MOELE+Indomethacin)	7.98+1.0[c]

LI*: Lesion index (Bands 4 mm in length was multiplied by 3, where 2-4 mm was multiplied by 2, and bands <2 mm multiplied by 1). Values are given as mean ± standard deviation (SD) for ten animals in each group. *Indicated significant differences at P<0.05 among control, indomethacin and groups 3 and 4. The different superscript letters (a, b, c) indicated a significant difference at P< 0.05, among groups 5 and 6 compared with group 2 (indomethacin).

Phytomedicinal agents have traditionally been used by herbalists and indigenous healers for the prevention and treatment of ulcers. The natural drugs were found to be the safer alternatives to cure ulcers. In this study the antiulcer activity of *M. oleifera* ethanolic leaf extract was evaluated in indomethacin-induced gastric ulcers in rats.

The results of the present study showed that MOELE possesses significant anti-ulcer activity, it showed a significant reduction in ulcer index (Table 1) compared to control (P<0.01). Indomethacin is known to produce erosions and ulcers in the stomach due to inhibition of cytoprotective prostaglandins (Vedavyasa, 1999).

Although, many products are used for the treatment of gastric ulcers, e.g. antacids and antihistamines; most of these drugs, however, produce several adverse reactions, like arrhythmias, impotence, gynecomastia and hematopoietic changes. Extracts of many herbal plants have been shown to produce promising results for the treatment of gastric ulcer (Verma et al., 2012).

MOELE was effective as a gastric cytoprotective agent; it may be due to its direct action on the mucus secretion or by increasing prostaglandins, thus protecting the stomach from indomethacin injury. It may be altering the antioxidant factors like total tissue sulfhydryl group (glutathione) suggesting that the healing of ulcers or prevention of the development of gastric ulcers in the model organisms, rats, is due to its antioxidant action.

The cytoprotective and antioxidant effects of MOELE may be contributed to the presence of some active phytochemical compounds such as alkaloids, sterols, glycosides, flavonoids, and terpenoids (Mahajan et al., 2008). Also, its leaves are rich in benzyl isothiocyanate which has anti-inflammatory activity (Lee et al., 2009).

In the present study, the antioxidant property, of 2 doses of *M. oleifera* leaf extracts exert its action via alteration in SOD, GPx, and MDA levels in rat gastric mucosa. During the ulcer condition, there is an increase in gastric mucosal SOD and lipid peroxidation (LPO) activities. This indicated that the generation of ROS during stress might be the causative factor for the inactivation of gastric peroxidase. *M. oleifera* leaf extracts exert their antioxidant defense mechanism probably by metabolizing lipid peroxides and scavenging endogenous H_2O_2 (Bhattacharya et al., 2000).

The superoxide anion (O_2^-), H_2O_2 and hydroxyl radical (OH^-) are the major ROS which induce cell degeneration by increasing lipid peroxidation of cell membrane lipids. The toxic end products of peroxidation induce damage of the structural and functional integrity of cell membranes, break DNA strands and denature cellular proteins. The natural cellular antioxidant enzyme includes SOD, which scavenges superoxide radicals by speeding up their dismutation.

Detoxification of the superoxide anion is not a terminating step in free radical scavenging, since the enzyme-catalysed dismutation results in the production of H_2O_2 which ultimately accumulates in the mitochondria and cytosol.

The results of the present study are similar to the finding of Mizui et al. (1986) which showed that the necrotizing substance like ethanol-induced gastric damage could be due to the formation of oxygen-derived free radicals resulting in lipid peroxidation and damage of cellular membrane with the release of intracellular component like lysosomal enzymes leading to further damage of leaves extract. *M. oleifera* was found to possess ulcer protective. Moreover, Moringa leaves contain isothiocyanate, which has anti-inflammatory as well as immune-modulatory activities (Shaila et al., 2010; Matsuda et al., 2007).

Biswas et al. (2012) reported that the presence of flavonoids in MOELE decreases the gut ulceration by improving microcirculation and increasing capillary resistance, so that the cells become more able to resist to the inflammatory factors.

LPO in the biological system has been demonstrated to be very important in mammalian physiology and pathophysiology. Increasing the rate of lipid peroxidation indicates the initiation of oxidative stress, which leads to various tissue injuries and cell death causing the

Table 2. Effects of *Moringa oleifera* ethanolic leaf extract (MOELE) on antioxidant levels in various groups in comparison with indomethacin-treated and control group.

Parameter/Group	MDA (nmol/100 mg protein)	GST (U/mg protein)	SOD (U/mg protein)	GPx (U/mg protein)
Group 1 (Control)	2.56+0.76	1.02+0.7	25.4 +1.34	20.1+1.5
Group 2 (Indomethacin)	6.6+1.12a*	0.43+0.01[a*]	12.6+1.4 [a*]	9.6+0.73 [a*]
Group 3 (100 mg-MOELE)	2.01+0.32	0.95+0.05	22.4+1.62	24.2+1.4
Group 4 (500 mg-MOELE)	1.99+0.09	1.87+0.14	31.2+1.97	28.6+1.8
Group 5 (100 mg-MOELE+Indomethacin)	4.01+1.05[b]	1.99+0.39[b]	17.3+1.05[b]	14.7+1.1[b]
Group 6 (500 mg-MOELE+Indomethacin)	3.03+0.81[b]	3.10+0.03[c]	26.4+1.09[c]	16.8+0.98[b]

Values are given as mean ±SD for ten animals in each group. *Indicated significant differences at P< 0.05 among control, indomethacin and groups 3 and 4. The different superscript letters (a, b, c) indicated a significant difference at P< 0.05, among groups 5 and 6 compared with group 2 (indomethacin).

progression of many acute and chronic diseases. The products of LPO such as malondialdehyde (MDA) are more cytotoxic to cells and have an effect on the membrane structure and function (Basu, 2003).

The results of the present study had shown that MOELE can restore the antioxidant activities of GST, GPx, and SOD and decrease the LPO which is induced by oral administration of indomethacin. Also, there was a notable decrease in gastric lesions. It is interesting to note that *M. oliefera* leaf extracts when given to healthy animals enhanced the level of antioxidants. The results could be explained by Sreelatha and Padma (2009) who conclude that, the leaves extract of moringa prevents oxidative damage to major biomolecules by scavenging the free radicals, so it can protect the biological cells against oxidative damage. Also, the presence of both vitamin C and A, can increase the efficiency of this plant in preventing the oxidative damage to the cell membrane of the biological cells (Bharali et al., 2003).

The current results are also in agreement with Devaraj et al. (2007) who observed that *M. oliefera* leaf extracts when given to normal animals enhanced the level of antioxidant condition. Verma et al. (2009) reported that the scavenging and antioxidant activities of *Moringa* leaves extract are due to the hydrogen proton donation of that compound.

The overall results of the present study are in consensus with the earlier observation of Bello and Balaraba (2012) who demonstrated that in stressed rats, *M. oliefera* leaves extract significantly attenuated the stress-induced gastric ulcerogenesis. Moreover, the leaves have quercetin, a flavonoid compound that is suggested to have a gastric cytoprotective effect and considered as antiulcer agents (Casa et al., 2000; Choudhary et al., 2013). In conclusion, the findings suggest a useful therapeutic activity for MOELE as an antioxidant and anti-ulcerative medicinal plant for gastric ulcer treatment. Oral administration of MOELE, even with low doses, blocks and disrupted free radical metabolism. The extract of *M. oleifera* ameliorated the ulcer lesion and SOD, catalase (CAT), and MDA levels in rat gastric mucosa due to an antioxidant property of MOELE. The antioxidant defense mechanism of the extract was probably due to metabolizing lipid peroxides and scavenging H_2O_2. More studies required to distinguish the exact mechanism and isolation and characterization of active ingredients from crude extract.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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Biodegradation of phenol by free and immobilized *Candida tropicalis* NPD1401

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The present research aimed to evaluate the free and immobilized cell of *Candida tropicalis* NPD1401 for phenol degradation. Immobilized cell of *C. tropicalis* degraded efficiently up to 98% at a concentration of 1000 mg/l of phenol whereas free cells degraded up to 63% of the same concentration under 9 days of incubation. Stored immobilized beads were reused after 15 days and found to have successfully degraded 62.1% of phenol in the mineral salt medium (MSM). Growth of *C. tropicalis* was observed in the phenol containing medium by measuring the dry weight of biomass (0.89 g/l at concentration 1000 mg/l) and the degradation was monitored using analytical techniques. Liquid chromatography-mass spectroscopy (LC-MS) analysis confirmed that phenol was degraded by ortho-pathways by the finding of metabolite *cis, cis*-muconic acid, phenyl phosphate and catechol. Next, isolated strain was identified on the basis of PCR amplification of sequence D2 region of the large subunit of 28S rDNA and it was confirmed as *C. tropicalis.* By observing the efficiency of the isolate it may be used for the further bioremediation purpose of the phenol contaminated site in the environments.

Key words*: Candida tropicalis,* phenol, ortho-pathway, Cis-cis-muconic acid, immobilized cell.

INTRODUCTION

Phenol is one of the major toxic aromatic compound discharges from industry and enters into the natural ecosystem. Phenol and phenol derivatives are released from petrochemical, chemical, pharmaceuticals, wood processing plants, paper and pulp, coke manufacturing and pesticide industries. Phenol is included as one of the most hazardous pollutants in the list of Environmental Protection Agency (EPA) (Pishgar et al., 2011). Phenol is also known as carbonic acid, phenic acid, phenylic acid, phenyl hydroxide and or oxybenzene (Nair et al., 2008). Inhalation and dermal exposure of phenol cause irritation, anorexia, progressive weight loss, diarrhea, vertigo, salivation, and a dark coloration of the urine (EPA, 2002). Repeated phenol exposure also causes renal damage, cardiovascular diseases and fatal for adult and children (ASTDR, 2014).

Considering the toxicity of phenol, it must be removed or its load must be reduced considerably from waste

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before its disposal in the environment. Treatment of waste containing phenol includes both physico-chemical and biological methods. Physico-chemical methods include adsorption, solvent extraction and chemical oxidation by ozone and chlorination (Molva, 2004). Nowadays, biological method is preferable compared to physico-chemical methods due to its expensiveness, and also generates toxic and non-biodegradable intermediate compounds. Microbial treatment of organic recalcitrant compounds is widely studied due to its potential of mineralization of toxic organic compounds. Various studied are carried out to degrade or metabolize the phenol and its derivatives into non-toxic, biodegradable compounds by using microorganisms such as bacteria, fungi and algae (Lika and Papadakis, 2009). Microorganisms have been isolated and studied for phenol and chlorophenols degradation capability such as *Acinetobacter, Alcaligenes, Corynebacterium glutamicum, Pseudomonas* sp*., Bacillus* sp*., Kocuria* sp*., Enterobacter* sp*.* and *Vibrio* sp*.* (Field and Sierra-Alvarez, 2008; Karn et al., 2010a,b, 2011, 2017, Karn and Geetanjali, 2014). Fungi are effective in degrading a wide range of organic molecules due to their release of extra-cellular enzymes and high biomass formation. Earlier, Chang et al. (1998) isolated and observed *Candida tropicalis* for the degradation of high concentration of phenolic and chlorinated derivatives compounds. Lika and Papadakis (2009) and Basha et al. (2010) also reported yeast sp. such as *C. tropicalis, Trichosporon* and *Rhodotorula* species; and mycelia as *Aspergillus niger, Phaenarochaetes chrysoporium* were used for the bioremediation of phenol. To achieve the successful remediation of particular compounds, selection of fungal species is important for degradation of phenol (Matsubara et al., 2006). Both aerobic and anaerobic processes were used to degrade phenol and its derivative, but aerobic process and microorganisms were found to be more effective in the treatment of phenolic pollutants (Al-Khalid and El-Naas, 2012).

In the last two decades, there have been exhaustive researches on the use of immobilized microbial cells as biocatalysts, Bacterial cells immobilized on various matrices have been used extensively for degradation of various toxic (Qi et al., 2012; Mulla et al., 2013). One of the limiting factors for phenol biodegradation is the concentration of phenol. Moreover, enzymes are accompanied by many other enzymes in microorganisms, sometimes with activities against the same substrate. A particular enzyme may be specific and selective, but if the contaminant enzymes have opposite (or just different) properties, this may reduce the apparent performance of the prepared biocatalyst (Palomo et al., 2002). To overcome this substrate limitation and increase the sustainability and reuse of microorganisms, immobilization of phenol degrading microorganisms was carried out in different immobilizing materials. Immobilization microorganism and enzymes are usual requirements for their large scale use (Santos et al., 2015). Considering these factors, the present work focused on the screening of efficient organism for phenol degradation by using free and immobilized culture to reduce the phenol concentration effectively.

MATERIALS AND METHODS

Isolation and screening of phenol degrading microorganism

Isolation of organism was done by enrichment method of the sludge sample collected from industrial waste site of Punjab Chemical, Lalru, Punjab, India (30.79° N 75.85° E), in the mineral salt medium. Next, 3 g of industrial sludge was taken and dissolved in 100 ml of mineral salt medium supplemented with phenol up to 1200 mg/l. The mineral salt medium contained the following components at the specified concentrations (in g/l): K_2HPO_4, 0.4; KH_2PO_4,0.2; $MgSO_4.7H_2O$, 0.2; $FeCl_3$,0.01; $CaCl_2.2H_2O$, 0.01; $MnSO_4.H_2O$, 0.01; Na_2MoO_4, 0.01; NaCl,0.1; glucose, 0.5; $(NH_4)_2SO_4$, 0.5. Further organism was isolated by using dilution plate techniques from dilution 10^{-5} to 10^{-7} on solid mineral salt medium plates were prepared by adding 15 g/l bacteriological grade agar and incubated at 28°C. Further, resistant organism was selected by successive culturing up to five generations.

Synthetic chlorophenol was purchased from Sigma Aldrich chemicals (USA) and other chemical reagents purchased were of analytical grade from Hi-Media, (India). All solutions were prepared in sterile Milli-Q water (Millipore direct Q3, Bangalore) India.

Growth, resistance and phenol degradation

The growth and phenol transformation response were conducted in 500-ml flasks, sealed with cotton stoppers, containing 100 ml of mineral salt medium (MSM) and inoculated with selected strains of *C. tropicalis* and were screened for their tolerance to phenol. Phenol was filter sterilized and added to the medium after autoclaving. One week old mycelia discs (2 x 5 mm inoculum) fungus disc cut from the actively growing mycelia were inoculated containing different concentrations (100, 200, 400, 800, 1000 mg/l) of phenol and incubated at 30°C with 120 RPM shaking. Control experiments using non-inoculated, sterile media with the same concentration and same conditions were also conducted. Phenol transformation was monitored at 10 days of incubation by collecting the 5 ml of sample. Growth was observed by means of biomass formation which was also harvested at 10 days of incubation, harvested biomass washed with distilled water, oven dried and the biomass was measured.

Immobilization of *Candida tropicalis*

2% (w/v) of the sodium alginate solution was dissolved in 25 mM Tris–acetate buffer (pH 7.5). The solution was stirred for 2 h at room temperature (25±2°C).The culture was centrifuged and the pellet was mixed into sodium alginate solution. The drops of this mixture were poured with the syringe into 100 ml of 3% (w/v) $CaCl_2$ solution which initiated the formation of beads. The solution was stirred for 90 min during calcium alginate bead formation. The collected beads were washed with 25 mM Tris-acetate buffer (pH 7.5) to remove excess Ca^{2+} and stored in the same buffer at 4ºC (Sivasubramanian and Namasivayam, 2014). Biodegradation of phenol by immobilized and free cells was studied at 1000 mg/l concentration of phenol. Reusability of immobilized cells was also evaluated after 15 days. Beads were stored at 4°C Tris-acetate buffer.

Phenol estimation by analytical methods and LC-MS analysis

Further analytical methods used for phenol estimation by 4-amino antipyrine were used as substrate for quantitative estimation of phenol by the spectroscopic method. Absorbance was measured at 510 nm (EPA, 2007) and it was further confirmed by liquid chromatography analysis and metabolic product was analyzed by LC-MS details described. LC-MS analysis of the sample was done by using a Waters Micromass Q-Tof Micro (the mass spectrometer is coupled with Waters 2795 HPLC). Sample culture was centrifuged at 5000 rpm for 20 min. Supernatant has been taken in fresh vial. Separation was achieved with an LC column Waters X-Terra C18 column, eluted with a gradient of acetonitrile in water containing acetic acid (0.1% v/v); from 0 to 40% acetonitrile, using the following parameters- ionization: electro spray positive (ES+), acquisition: MRM unit resolution, Injection volume: 20 □l and Flow rate: 0.15 ml/min. For mass spectrometer, the following parameters were used: desolvation gas: 550 L/h; cone gas: 30 L/h; desolvation temperature: 250°C; source temperature: 110˚C; capillary voltage: 3000 V; cone voltage: 30 V; collision energy: 4 eV; nebulize gas: nitrogen 30 ml/min; collision gas: argon 0.5 µl /min (Comte et al., 2013; and Glish and Vachet, 2003*).*

Identification of phenol degrading strain

Fungal gDNA Miniprep Kit (XcelGen, Gujarat, India) was used for the isolation of genomic DNA. The quality of DNA was evaluated by electrophoresis on 1.2% agarose gel. A fragment of D2 region of 28S rDNA gene was amplified by PCR from the isolated genomic DNA. Reaction mixture for the PCR contained 1X PCR buffer; 200 µM of dNTPs; 1.5 mM $MgCl_2$, 0.1 µM of each primer and 2.5 units of Taq DNA polymerase (Invitrogen, USA) in a final volume of 100 µl sterile MQ water. The PCR was performed with initial denaturation carried out at 95°C for 4 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C and extension at 72°C for 30 s. The thermal cycler was terminated by a final extension for 5 min at 72°C. The sequence for DF/DR primer was as follows: DF: 5'-ACCCGCTGAACTTAAGC-3', and DR: 5'-GGTCCGTGTTTCAAGACGG-3' (Fell, 1993). The PCR amplicon was purified and further processed for the sequencing. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with DF and DR primers using BDT v3.1 Cycle sequencing kit on ABI 3730xlgenetic analyzer. The D2 region of 28S rDNA gene sequence was used to carry out BLAST with the nr-database of NCBI Genbank database. Based on maximum identity score, 15 sequences were selected and the phylogenetic tree was constructed using MEGA 7.

Data analysis

Data were statistically analyzed by analysis of variance (ANOVA) and the mean differences were compared by Tukey-Kramer Multiple Comparison Test at $p < 0.05$. All the experiment was conducted with three replicates and the analyses were performed using GraphPad Prism (v 4.03) software.

RESULTS AND DISCUSSION

Isolation, screening and identification of phenol degrading microorganism

Resistant fungal strains was screened; out of seven isolates just only one isolate (NPD1401) was able to grow at 1200 mg/l of phenol on minimal salt agar plate therefore, this strain was selected for further study. The selected strain was identified by molecular technique, by D2 region of 28S rDNA gene using PCR. A single band of 700 bp of D2 region of large subunit 28S rDNA has been obtained as shown in Figure 1 and sequenced. Consensus sequence of 638 bp of D2 region of LSU gene was generated from forward and reverse sequence data using MultAlin Program (http://bioinfo.genotoul.fr/multalin/multalin.html) and alignment was manually corrected. Furthermore, based on the BLAST search analysis, strain NPD1401 showed 99% similarity with *C. tropicalis.* The obtained sequence of *C. tropicalis* was submitted to GenBank (NCBI) with accession number KT380847.

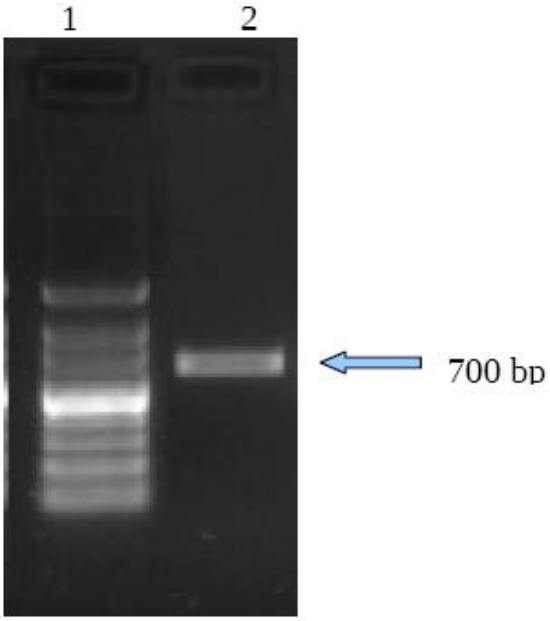


Figure 1. 1.2% Agarose gel showing single 700 bp D2 region of 28S rDNA amplicon band. Lane 1, DNA marker (1kb ladder); lane 2, D2 region of 28S rDNA amplicon band of *Candida tropicalis.*

The evolutionary relationship was inferred using the Neighbor-Joining method. The bootstrap consensus tree was inferred from 1000 replicates (Kimura, 1980). The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 583 positions in the final data-set. Further phylogenetic were constructed using MEGA7 software (Kumar et al., 2016) and shown in Figure 2. Recently, Karn et al. (2017) also isolated pentachlorophenol (PCP) degrading straion SK1 by enrichment method and identified by same method discussed in the current study.

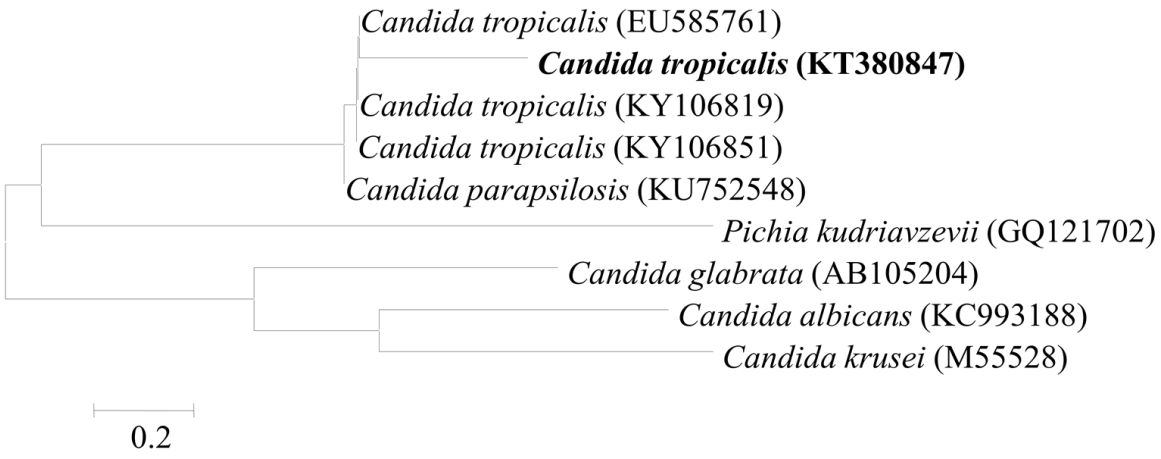


Figure 2. N-J tree based on 28S rRNA sequence of current study *Candida tropicalis* (KT380847) along with sequences available in GenBank database which shows close similarity with *Candida tropicalis.*

Table 1. Growth observed in terms of dry weight (g/l) and degradation (in %) by *Candida tropicalis* at different concentration of phenol.

Time (Days)	**Dry wt. observed in (g/l) of *Candida tropicalis* at different concentration of phenol by free cell**					
Concentration	0.00	100	200	400	800	1000
Time(10 Days)	0.00	1.57±0.02^{A}	1.51±0.06^{A}	1.33±0.06^{B}	1.29±0.03^{B}	0.89±0.05^{C}

Time (Days)	**Degradation observed at different concentration of phenol by free cell in %**					
Concentration	0.00	100	200	400	800	1000
Time (10 Days)	0.00	99±2%^{A}	98±4.2%^{A}	81±1.5%^{B}	72±2.7%^{C}	63±1.7%^{D}

*Value sharing common uppercase letter within row are not significant at $p < 0.05$ value. Data are mean and standard deviation of triplicate.

Biodegradation of phenol by free and immobilized cells

During degradation the well grown biomass in the MSM was observed and after completion of 9 days the dry weight given in Table 1 was observed. *C. tropicalis* has shown effective degradation up to 200 mg/l degradation was about 99%, at 400 mg/l it was 81%, at 800 it was about 72%, and 1000 mg/l it was about 63% using the free cell within ninth day of incubation (Table 1). By observing the efficiency of this isolate, we directly used 1000 mg/l for immobilized and the fresh immobilized cells degraded 98.01% of phenol within 8 days. The immobilized beads of isolates were stored on 25 mM Tris-acetate buffer at 4°C for 15 days. Further, it was re-used and an observed 62.3% of phenol was degraded by stored immobilized cells within 9 days of incubation (Figure 3). *C. tropicalis* NPD 1401 strain was shown as efficient phenol degrading strain. Previously, Rocha et al. (2007) isolated *C. tropicalis*, *C. rugosa*, and *Pichia membranaefaciens* strain; of these three strains, only *C. tropicalis* was capable of growing at higher phenol concentration, that is, 1000 mg/l in the minimal medium. Zhou et al. (2011) designed statistical experiment and used optimized process of phenol degradation by *C. tropicalis* Z-04. The predicted results showed that the maximum removal efficiency of phenol (99.10%) could be obtained under the optimum conditions of yeast extract 0.41 g/l, phenol 1.03 g/l, inoculum size 1.43% (V/V) and temperature 30.04°C. These predicted values were further verified by validation experiments. Wang et al. (2012) used *C. tropicalis* W1 isolated from the sludge in the Yantai River of China by selective enrichment with phenol and investigated degraded phenol concentration of 900 mg/l in 30 h, but had no marked degradation activity in 4-chlorophenol. Sivasubramanian and Namasivayam (2014) also observed bioremediation of phenol using *C. tropicalis* SSK01 immobilized cells isolated from petroleum contaminated soil and observed maximum phenol degradation was 95.2% degradation at 34.20°C and pH 6.86 with a concentration of 610 mg/l. Due to the efficiency towards the degradation of *C. tropicalis,* various researchers focused on this species. By comparing the previous isolates for phenol

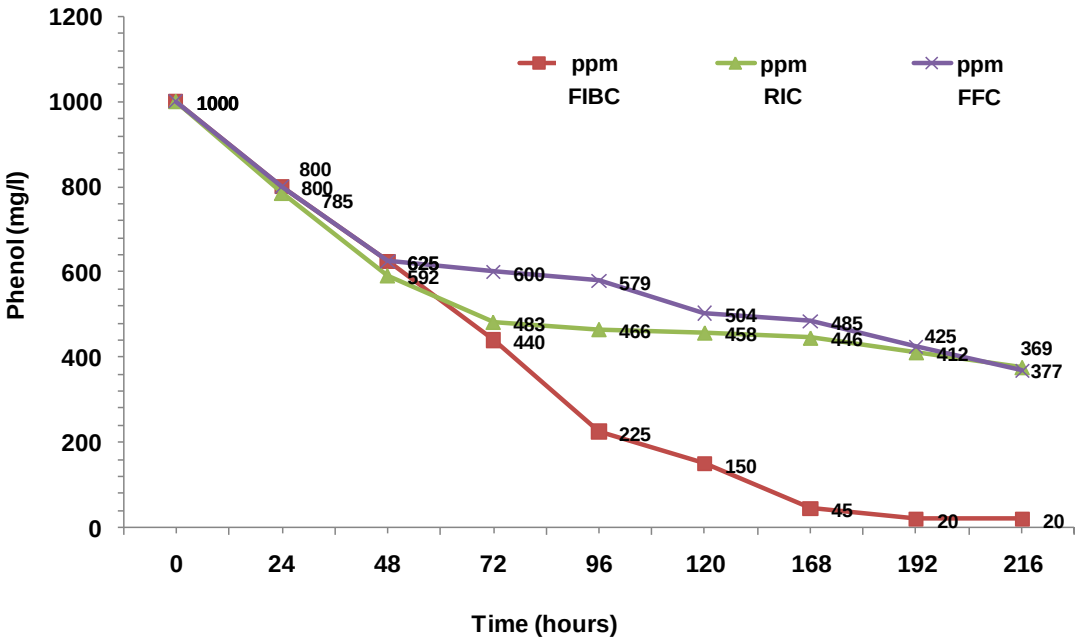


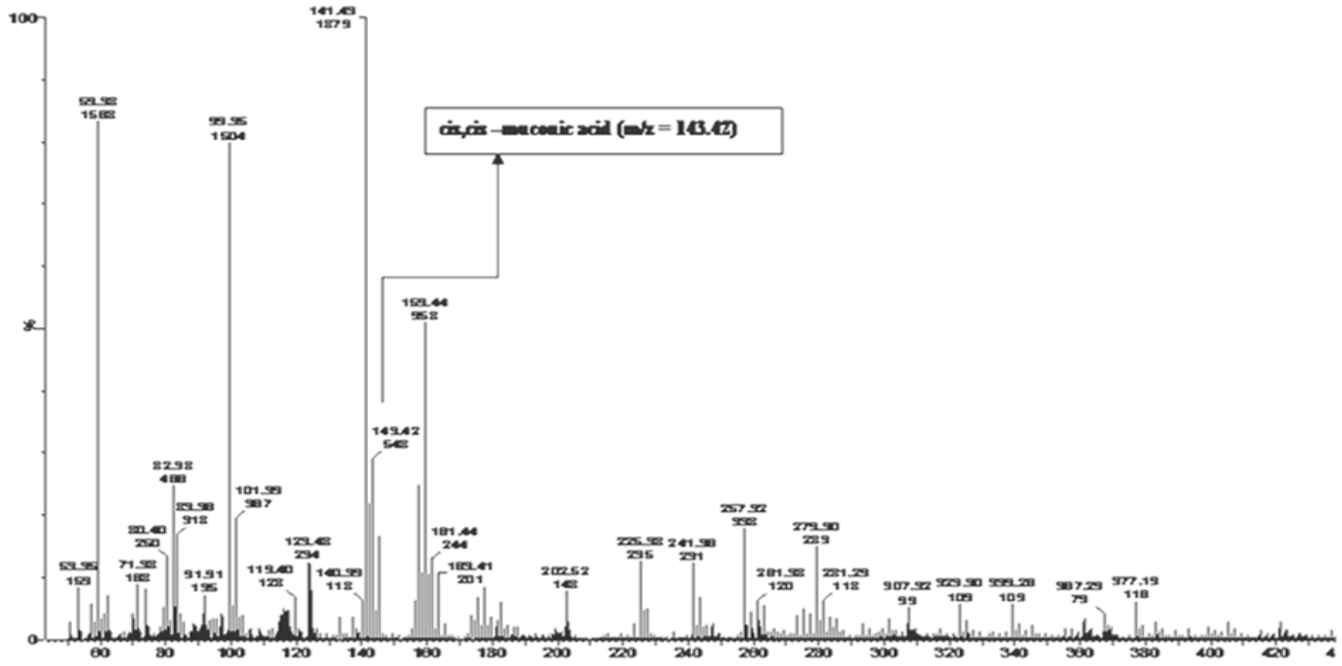
Figure 3. Comparative biodegradation of phenol by *Candida tropicalis* NPD1401. FIBC, Fresh immobilized bead cells; RIC, reused immobilized cells; FFC, fresh free cells.

degradation, it was observed that the current strain is a more efficient and degraded higher concentration of phenol. Among the various species of yeast, *C. tropicalis* is the most studied yeast species for its potential for phenol degradation (Yan et al., 2005; Adav et al., 2007; Zhou et al., 2011; Ahmad et al., 2013; Basak et al., 2014a; Long et al., 2014). Besides *C. tropicalis*, other yeast such as *C. lipolytic*a, *Candida utilis*, *Candida albicans*, *Trichosporon montevideense*, and *Trichosporon cutaneum* were also used to degrade the phenol and its derivatives (Chen et al., 2002; Vilimkova et al., 2008; Liu et al., 2011; Gerginovaa et al., 2014). Compared with free cells, immobilized cell was found more efficient for phenol degradation. Previously, polyacrylamide (PAA) gel beads, calcium alginate beads, sugarcane bagasse, agar-entrapment by Ramírez et al. (2001), Basak et al. (2014b) and Adav et al. (2007) were used to immobilize *C. tropicali*s and degrade different concentration of phenol. Aerobic granules of *C. tropicalis* were sufficient enough to degrade the phenol up to 1000 mg/l. The highest concentration of phenol (>1000 mg/l) was inhibitory for *C. tropicalis* present in the aerobic granule (Adav et al., 2007). Vilimkova et al. (2008) found NADPH-dependent phenol hydroxylase and catechol-1, 2-dioxygenase from *C. tropicalis* which helps in the degradation of phenol. The present strain successfully degraded phenolin both free as well as immobilized cell.

Radovich (1985) demonstrated mass transfer limitations caused by the transport resistance within the immobilization matrix affect the activity of the immobilized cells. A concentration gradient within the immobilized cell matrix (ICM) is established at steady state. Assuming that the distribution of cells is such that the fermentation reaction occurs throughout the ICM, the process must be modeled as simultaneous reaction and diffusion. The internal mass transfer effects are also traditionally accounted for by an effectiveness factor, which is defined as the ratio of the actual reaction rate to the reaction rate which would occur if all the interior of the biocatalyst particle was exposed to the same reactant concentration as the exterior of the particle. These mass transfer limitations may bring about an inhomogeneous distribution of viable cells within the immobilizing matrix, a change in the cell's growth kinetics or the cell's enzyme kinetics, and a change in the operational stability of the cells. Homaei et al. (2013) suggested that the heterogeneity of the immobilized enzyme systems allows an easy recovery of both enzymes and products, multiple re-use of enzymes, continuous operation of enzymatic processes, rapid termination of reactions, and greater variety of bioreactor designs.

LC-MS analysis

After LC-MS analysis, we determined that possibly phenol degradation is initiated in the presence of molecular oxygen and the aromatic ring is further hydrolyzed by the phenol hydroxylase into catechol. The aromatic ring of phenol breakdown by the ortho or meta-oxidation pathways was described previously by Jiang et al. (2006), and Lika and Papadakis (2009). Catechol 1, 2-



(a)

Figure 4a. LC-MS analysis of phenol. **a)** Cis,cis-muconic acid as metabolite. **b)** Phenyl phosphate and catechol.

dioxygenase and catechol 2, 3 dioxygenase are the enzymes involved in the breakdown of aromatic ring present in the phenol by ortho and meta pathway respectively, with the cleavage sites for both enzymes different (An et al., 2001; Cai et al., 2007; Nair et al., 2008; Agarry et al., 2008). Wang et al. (2007) reported on the *Acinetobacter* sp. PD12 metabolized phenol in the o-pathway and detected the presence of catechol 1, 2-dioxygenase. In the result, we found featured peak (Figure 4a) having m/z 143.42 which showed the presence of *cis, cis* - muconic acid. During phenol degradation *cis, cis* -muconic acid was prominently detected using LC-MS analysis (LC-MS of the sample was analyzed and outsourced at SAIF, Punjab University, Chandigarh, India). The cis,cis muconic acid belongs to aerobic degradation ortho-pathways for phenol biodegrdation. Due to the presence of *cis, cis* - muconic acid it has been predicted that *C. tropicalis* used *ortho*-metabolic pathway for phenol degradation. Presence of *cis, cis*-muconic acid indicates the isolated yeast strain and follows *ortho*-metabolic pathway for biodegradation of phenol. The present result was supported by the finding of Tuah (2006) who also confirmed that *C. tropicalis* strain follows *ortho* - metabolic pathway for phenol degradation. The enzymes involved in metabolic pathways are specific for substrate used. Other intermediate metabolite of degraded phenol like phenol pyrophosphate and catechol (Figure 4b) which provide evidence of the ability of *Candida tropicalis* for phenol degradation was also observed. Thus, we can clearly say that the present strain are efficient for phenol degradation and can be applied to the contaminated site in the environment.

Conclusion

The present research comes up with efficient strain which degraded phenol 98% by immobilized culture and free cell 63% effectively at high concentration in 9 days incubation period. Further, LC-MS finding revealed its metabolite cis-cis muconic acid and catechol clearly evidenced for the degradation of phenol. Further application of this strain for phenol remediation in real contaminated environment is underway.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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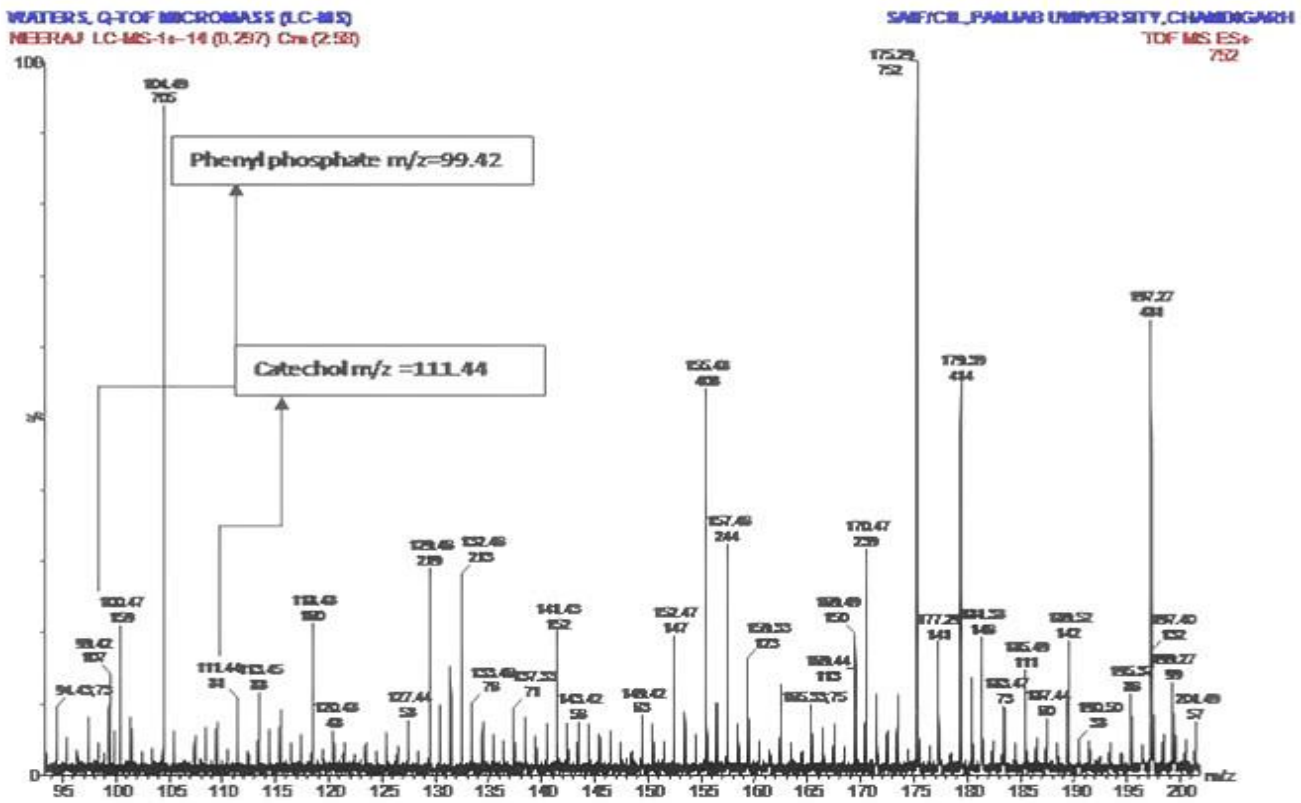

(b)

Figure 4. Cont'd.

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