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ANTIBACTERIAL MODE OF ACTION OF BIO-OIL OBTAINED FROM PYROLYSIS OF *PINUS DENSIFLORA* SIEBOLD & ZUCC. SAWDUST AGAINST FOOD POISON CAUSING *STAPHYLOCOCCUS AUREUS* ROSENBACH

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Abstract

Staphylococcus aureus is a major foodborne pathogen that causes food poisoning. The antibacterial potential of pyrolyzed bio-oil (PBO) manufactured from sawdust of *Pinus densiflora* against three strains of pathogenic *S. aureus* (ATCC 49444, ATCC 12600 and ATCC 12692) was evaluated for its mode of action. PBO at 1,000 µg/disc exhibited strong antibacterial activity against *S. aureus* ATCC 49444 and ATCC 12600, with 10 - 11 mm inhibition zones. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of PBO ranged from 250 to 500 and 500 to 1,000 µg/ml, respectively. The mode of action of PBO at the MIC against *S. aureus* ATCC 49444 revealed its strong impairing effect on membrane permeability in terms of its increased relative electrical conductivity, release of 260 nm absorbing material and leakage of potassium ions (K^+), along with its loss of high salt tolerance capacity. Overall, the results of this study suggest that PBO exerts a strong antibacterial effect against *S. aureus* via disturbance of membrane integrity.

Introduction

Food poisoning due to contamination by different types of microorganisms is a major problem worldwide that affects millions of people each year (Oussalah *et al.* 2007, da-Silveira *et al.* 2012). Although there have been many advances in the field of sanitation techniques and food processing procedures, the contamination of food cannot be completely eradicated during food processing, storage and distribution (Runyora *et al.* 2010). Foodborne bacteria are responsible for various food-related diseases; however, their modes of infection vary among species (Loir *et al.* 2003). Some bacteria are directly responsible for food contamination and spoilage via their direct infection through bacterial spores, while others act indirectly via the production of toxins resulting in food-borne poisoning, causing symptoms ranging from gastrointestinal disorders to paralysis and death (Loir *et al.* 2003).

Staphylococcus aureus is a major foodborne bacterium that is the causative agent of food poisoning because of its toxin production (Loir *et al.* 2003). *S. aureus* primarily contaminates food products during preparation and processing (Loir *et al.* 2003, da-Silveira *et al.* 2012). Recently, bacterial pathogens were found to be resistant to classical preservatives and antibacterial agents (Militello *et al.* 2011). Thus, protection of food from this microorganism is a constant struggle for food industries, which are continuously engaged in development of a variety of techniques and agents to control the pathogen (Bajpai *et al.* 2013).

Consumers have become aware of the quality and safety of processed foods and thus demand highly nutritious, tasty and natural foods (Bajpai *et al.* 2013). There is also great demand for restriction of the use of synthetic preservatives that have conventionally been used in foods for

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decades and cause various health-related diseases (Burt 2004, Silva *et al.* 2011). To meet these increasing consumer demands, modern food processing industries are continuously engaged in a search for alternatives to synthetic chemicals and additives. Different types of plants and their products are continuously being tested for use in food preservation and as additives (Fabian *et al.* 2006, Silva *et al.* 2011).

Application of plant based natural bio-oil (BO) as an antibacterial agent could be an alternative source of natural bioactive compounds that can act against multi-resistant *S. aureus*. Normally, bio-oils are manufactured from woody biomass and agricultural residues by thermochemical pyrolysis to convert them into liquid bio-oil together with bio-char and gases. In this process, organic materials from the biomasses are degraded under high temperature to form BO (Patra *et al.* 2015a). These plant based BOs could be an alternative source of antimicrobials against pathogenic bacteria responsible for food related diseases. The antibacterial properties of bio-oils against some pathogenic microbes have been reported (Bedmutha *et al.* 2011, Phukan *et al.* 2013, Hossain *et al.* 2014). Moreover, pyrolyzed bio-oil (PBO) from the sawdust of Japanese red pine was shown to possess a number of bioactive compounds including alcohols, acids, ethers, phenols, and phenol derivatives (Kim *et al.* 2011, Patra *et al.* 2015b). Trimethyl orthoacetate, 4-oxo-5-methoxy-2-penten-5-olide, 1,4-methanoazulene, and benzene methanamine are some of the dominant compounds present in PBO (Patra *et al.* 2015b). These bioactive compounds present in PBO could serve as a potential source of natural antibacterial agents against pathogenic *S. aureus*. Therefore, the antibacterial potential of PBO against different pathogens of *S. aureus* was investigated, along with the mode of its antibacterial action.

Materials and Methods

Plant bio-oil (PBO) was manufactured from the sawdust of Japanese pine wood (*Pinus densiflora* Siebold and Zucc.) by fast pyrolysis using a lab-scale fluidized-bed fast pyrolyzer as previously described (Hwang *et al.* 2013, Patra *et al.* 2015b). Prior to the manufacture of PBO, the sawdust was air dried to approximately 7% moisture content and ground to powder (particle size of 0.5 mm) followed by the fast pyrolysis at 500°C with a residence time of 1.3 sec. Black oil like liquid obtained was kept in air tight glass bottles at 4°C until further use.

In vitro antibacterial activity of PBO was evaluated against three foodborne pathogens, *S. aureus* ATCC 49444, *S. aureus* ATCC 12600 and *S. aureus* ATCC 12692, using the standard disc diffusion method (Patra *et al.* 2015a). The foodborne pathogens were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Prior to use, all three pathogenic bacteria were sub-cultured in nutrient broth media (NB) from Difco (Becton, Dickinson and Company, Sparks Glencoe, MD, USA). After 1 ml of standardized inoculum containing 10^7 cfu/ml of bacterial suspension was spread uniformly on nutrient agar (NA) plates, 6 mm sterile filter paper discs (Advantec, Toyo Roshi Kaisha Ltd., Japan) impregnated with 1,000 µg of PBO/disc were placed on the plates. Kanamycin (Sigma-Aldrich Co., St. Louis, MO, USA, 40 µg/disc) and 5% dimethylsulphoxide (DMSO) was used as the positive and negative control, respectively. Antibacterial activity was evaluated after incubation at 37°C for 24 hrs by measuring the diameters of the zone of inhibition against the tested bacteria.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by the two-folds serial dilution method as previously described (Patra *et al.* 2015a). Different dilutions of PBO in 5% DMSO at concentrations of 2000, 1000, 500, 250 and, 125 µg/ml were prepared by the two-folds dilution method in NB in a 96 well microplates and to them, 10 µl of the test pathogens were added and incubated at 37°C for 24 hrs. The lowest concentration of PBO that did not show any visual growth of the tested pathogens on the

microplates was determined as the MIC value whereas lowest concentration of PBO that displayed complete absence of growth of bacterial pathogens in the form of colonies on the surface of NA plates was determined as the MBC values.

To elucidate the antibacterial mode of action of PBO against *S. aureus*, the *S. aureus* ATCC 49444 strain was selected. The effects of PBO on the viability of the bacterial cells were evaluated according to the standard procedure described by Patra *et al.* (2015c). For the viable cell assay, bacterial cells treated with PBO at the MIC were used as the treatment and bacterial cells treated with 5% DMSO were used as the control. The effects on viability were monitored for 8 hrs at a 2 hrs time interval. The results were compared with those of the control sample and expressed as $\log_{10}$ cfu/ml.

The effects of PBO on the membrane permeability of the tested foodborne bacteria were determined according to the method described by Kong *et al.* (2008). Before starting the assay, *S. aureus* ATCC 49444 was grown at 37°C for 10 hrs. Bacteria treated with the MIC of PBO were taken as the treatment and bacteria treated with 5% DMSO were taken as the control. The results were expressed in terms of relative electrical conductivity measured using a conductivity meter (Con 6, LaMotte, MD, USA) at 2 hrs time interval during 8h of incubation at 37°C in an incubator. The permeability of the bacterial membrane was calculated using the following equation:

$$\text{Relative conductivity (\%)} = \frac{(2 - L_1)}{L_0} \times 100$$

where, L_0 is the electrical conductivity of killed-bacteria in 5% glucose being treated in boiling water for 5 min, L_1 is the electrical conductivity of PBO at the MIC added to 5% glucose and L_2 is the electrical conductivity of bacteria treated with PBO at 2 hrs time intervals during 8 hr of incubation.

The release of 260 nm absorbing cellular materials from *S. aureus* ATCC 49444 after treatment with MIC of PBO was measured using the method described by Carson *et al.* (2002). Bacterial culture treated with the MIC of PBO was taken as the treatment and bacterial culture in sterile peptone water treated with 5% DMSO was taken as the control. Both the control and treatment bacterial culture were incubated at 37°C, and the absorbance of the supernatant after centrifugation at 3,500 rpm was measured every 30 min during 2 hrs of incubation. The results were expressed in terms of the optical density (OD_{260}) at 260 nm.

The leakage of potassium ions (K^+) from *S. aureus* ATCC 49444 treated with PBO was determined as per the standard procedure (Lee *et al.* 2002). Bacterial culture treated with the MIC of PBO was taken as the treatment and bacterial culture treated with 5% DMSO was taken as the control. Both the control and treatment bacteria were incubated at 37°C, and the concentration of free K^+ ions in bacterial suspensions was measured every 2 hrs during 8 hrs of incubation by a photometric procedure using the Kalium/Potassium kit (Quantofix, GmbH, Wiesbaden, Germany). Results were expressed in terms of the amount of free extracellular K^+ in mg/l released at each sampling interval.

The salt tolerance of *S. aureus* ATCC 49444 treated with PBO was investigated according to the method described by Miksusanti *et al.* (2008). Bacterial culture treated with the MIC of PBO was taken as the treatment and the bacterial culture treated with 5% DMSO was taken as the control. Both control and the treatment cultures were grown in NA plates supplemented with different concentrations (0, 2.5, 5 and 10%) of NaCl. The mean proportions of survivors in terms of $\log_{10}$ cfu/ml from treated suspensions were compared to the corresponding means for the untreated controls. The results of the experiments were expressed as the mean ± standard deviation (Sd). Statistical analysis to test the significance differences was conducted using ANOVA and

Duncan test, with a $p < 0.05$ considered to indicate significance. All analyses were conducted using Statistical Analysis Software (SAS) version 9.4 (SAS Inc., Cary, NC, USA).

Results and Discussion

Unlike other bacteria, contamination with *Staphylococcus aureus* can be readily avoided by heat treatment of food, but it still remains a serious problem because its toxins cause various food-related diseases, and can even be lethal (Loir *et al.* 2003). *S. aureus* can grow in a wide range of temperatures (7 - 48°C) and pH (4 - 9), which enables it to grow in a wide variety of food products (Bergdoll 1989). For these reasons, this bacterium has become an important pathogen to the food industry (Loir *et al.* 2003). Moreover, consumers have become aware of the safety and health-related issues caused by the use of synthetic and chemical food additives, which has led to increased demand for safer and natural treatments for ready to eat foods. As a result, the food industry has searched for alternative sources of natural antibacterial agents to be used in processing and preservation of food that can act effectively against multi resistant *S. aureus.* Various plants and their products have long been used for processing and preservation of food (Balchin and Deans 1997). In the present study, use of PBO manufactured from the fast pyrolysis of sawdust of Japanese red pine was tested as a natural antibacterial agent against different strains of pathogenic *S. aureus* with the elucidation of the mode of antibacterial action.

The results of the antibacterial activity of PBO against three different foodborne pathogens of *S. aureus* is presented in Table 1. PBO at a concentration of 1,000 µg/disc was effective against only two of these strains (ATCC 49444 and ATCC 12600), with diameter of zones of inhibition ranging between 10.0 and 11.0 mm, whereas it did not show any positive activity against *S. aureus* ATCC 12692 (Table 1). Kanamycin, the positive control, showed higher activity (16.0 - 17.6 mm zone of inhibition) than PBO against all the tested three strains (Table 1). The MIC and MBC values of PBO against the two pathogens varied between 250 and 500 µg/ml and 500 and 1,000 µg/ml, respectively (Table 1). Earlier reports suggested that plant-based pyrolysis oils possess certain groups of active compounds that are effective against a group of pathogenic microorganisms (Bedmutha *et al.* 2011, Phukan *et al.* 2013). Chemical analyses of different types

Table 1. Antibacterial activity, MIC and MBC of PBO against *Staphylococcus aureus.*

Bacterial strain	PBO*	Kanamycin**	MIC***	MBC***
S. aureus ATCC 49444	$11.0 \pm 1.0^{b\#}$	17.6 ± 2.52^{a}	250	500
S. aureus ATCC 12600	10.0 ± 0.0^{b}	16.0 ± 1.15^{a}	500	1000
S. aureus ATCC 12692	0 ± 0^{c}	16.6 ± 2.51^{a}	0	0

*Concentration of PBO at 1,000 µg/disc; **Standard antibiotic at 40 µg/disc; ***MIC and MBC in µg/ml; #Data are expressed as the means ± Sd; Values with different superscripts are significantly different at $p < 0.05$.

of pyrolysis oils have shown the presence of phenolic and other active compounds (Kim *et al.* 2011, Kim *et al.* 2012). Our previous study on the chemical analysis of PBO by GC-MS analysis, has revealed the presence of various active compounds such as 1,4-methanoazulene, 4-oxo-5-methoxy-2-penten-5-olide, benzenemethanamine, p-cresol, and trimethyl orthoacetate in higher quantity along with a number of alcohol, acids, aldehydes, alkanes, benzenes, furans, and naphthalenes etc. (Patra *et al.* 2015b). Thus the presence of these active compounds in the PBO might have been responsible for its antibacterial potential against the dreadful foodborne pathogenic bacteria.

The antibacterial mode of action of PBO against *S. aureus* was evaluated by various assays, including effects on cell viability, cell membrane permeability, release of 260 nm absorbing material and leakage of K^+ ions, as well as loss of the ability to tolerate high salt concentrations.

To accomplish this, *S. aureus* ATCC 49444 was selected as the model organism because it has the highest antibacterial selectivity. Upon exposure of *S. aureus* ATCC 49444 to PBO at the MIC, there was a gradual decrease in the number of cfu after 4 hrs of incubation and no growth of bacteria after 6 hrs of incubation (Fig. 1A). These findings suggest that PBO has antibacterial effects against *S. aureus* and leads to complete lysis of the bacterial cells after 6 hrs of incubation. A possible reason for the activity of PBO against *S. aureus* might be due to its hydrophobic nature, which would enable it to penetrate the cell membrane of Gram positive *S. aureus* ATCC 49444, eventually causing deformities and cellular lysis (Sikkema *et al.* 1994). The bioactive compounds present in PBO after entering the bacterial cell membrane could have affected the cell membrane leading to cellular lysis.

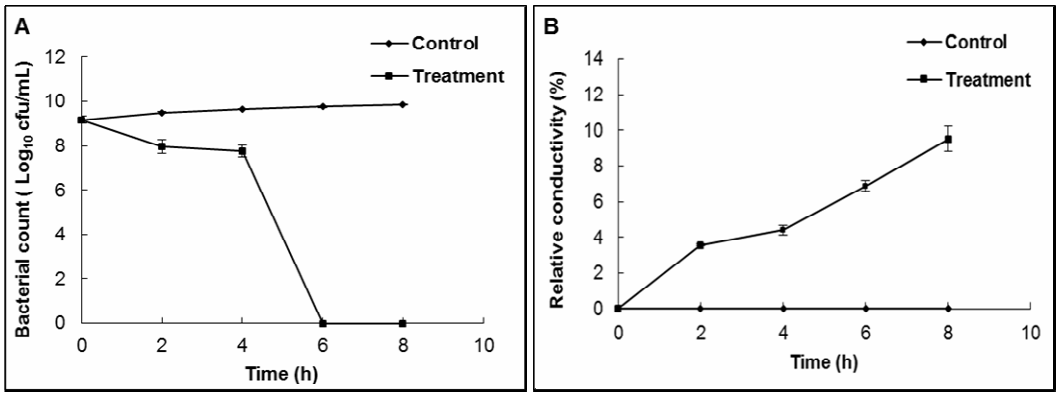

Fig. 1. Effect of PBO on the viability (A) and cell membrane permeability (B) of *Staphylococcus aureus* ATCC 49444. Data are expressed as the means ± Sd (n = 3).

The effects of PBO on the cell membrane of *S. aureus* ATCC 49444 are presented in Fig. 1B. The results indicated that there was a continuous increase in the relative electrical conductivity of bacteria treated with PBO at the MIC with time, whereas under same condition the control bacteria created with DMSO did not show any relative conductivity (Fig. 1B). Similarly, the increase in the release of 260 nm-absorbing materials to the outer bacterial solution was also observed only in PBO-treated bacterial cells (Fig. 2A).

As shown in Fig. 2B, treatment of *S. aureus* ATCC 49444 treated with the MIC of PBO induced leakage of intracellular K^+ ions to outside, especially after 6 hrs of incubation, whereas treatment with 5% DMSO did not induce K^+ ion leakage. The cytoplasmic membrane of the bacterial cell controls the entry and exit of Ca^{2+}, H^+, K^+ and Na^+ ions, and thus maintains various cellular functions like maintenance of the energy status, solute transport, regulation of metabolism, control of turgor pressure and energy-transuding processes inside the bacterial cell (Cox *et al.* 1998). However, the effects of PBO caused a disturbance in the integrity and normal metabolic activity of pathogenic *S. aureus* ATCC 49444 that might have been the reason for the cellular death of the bacterium caused due to the leakage of the cell membrane and release of 260 nm absorbing materials, small ions and proteins from the cell (Cox *et al.* 2000, Zhu *et al.* 2005). The results of the present investigation are in agreement with earlier studies reporting that many

antibacterial compounds act by facilitating leakage of the cytoplasm and its coagulation, which affected the integrity and function of the cell, ultimately leading to death (Denyer 1990, Cox *et al.* 2000). The internal environment of the bacterial cell is known to be rich in various small ions such as K^+, and leakage of this ion into media from PBO-treated bacteria confirmed that its membranolytic effects caused cellular leakage (Cox *et al.* 2000).

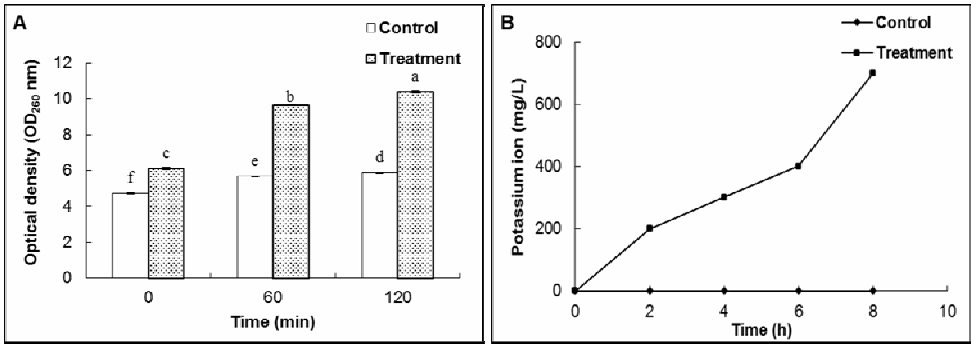

Fig. 2. Effect of PBO on the release rate of 260 nm absorbing materials (A) and leakage of potassium ions (B) from *Staphylococcus aureus* ATCC 49444. Data are expressed as the mean ± Sd (n = 3). Values with different superscripts are significantly different ($p < 0.05$).

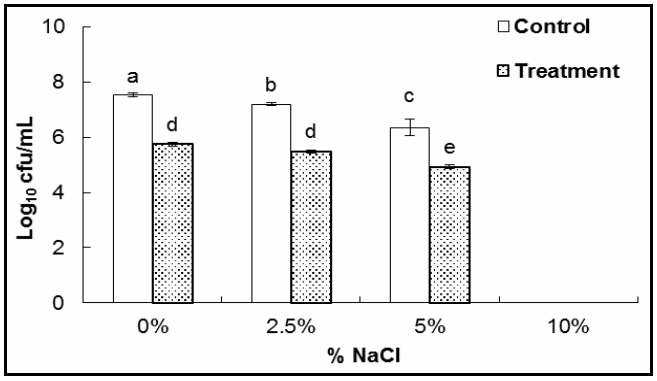

Fig. 3. Effect of PBO on loss of salt tolerance capacity of *Staphylococcus aureus* ATCC 49444. Data are expressed as the means ± Sd (n = 3). Values with different superscripts are significantly different ($p < 0.05$).

The loss in salt tolerance of *S. aureus* ATCC 49444 in response to treatment with PBO at the MIC is presented in Fig. 3. When bacteria treated with PBO or DMSO were cultured on NA plates supplemented with different concentrations (0, 2.5, 5 and 10%) of NaCl, there were less PBO-treated bacteria on all NA plates than DMSO-treated bacteria, regardless of salt-concentrations (Fig. 5). There was also a gradual reduction in the number of cfu in response to higher concentrations of salt among both PBO- and DMSO-treated *S. aureus* ATCC 49444 (Fig. 5). This might indicate that PBO could have made sublethal injuries to bacterial cell membranes, causing loss of their ability to osmoregulate the cell adequately or to exclude toxic materials, ultimately resulting in death of the bacteria (Carson *et al.* 2002, Miksusanti *et al.* 2008).

In the present investigation, PBO manufactured from the sawdust of Japanese red pine displayed potent antibacterial activity against food poisoning *S. aureus*. Discrete studies of the mode of its antibacterial action revealed that PBO acted on *S. aureus* by affecting the membrane integrity of the bacterial cell, causing membrane leakage that leads to cell death.

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