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Review

Microorganisms in functional food supplementation: A review

Karina Teixeira Magalhães-Guedes^{1*}, Camila Duarte Ferreira¹, Kassiana Teixeira Magalhães², Rosane Freitas Schwan³, Jorge Alberto Vieira Costa⁴ and Itaciara Larroza Nunes¹

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Use of microbial systems for functional foods supplementation can be defined into fermentative process by starter cultures, use of probiotics microorganisms and use of microbial biomass. Scientific and technical aspects as related to use of microorganisms in functional foods supplementation are highly diverse and complex, since they have to deal with the viability of probiotic strains as well as with the safe production, nutritional composition and acceptability of the new product. However, limited information is available on the microorganisms which can be used in functional food supplements, as well as how to use these microorganisms and generation of products. This review reports details of this information. The data from this review can be useful to support the development of new functional products, with microbial supplementation, for the market.

Key words: Kefir, yeast, microalgae, lactic acid bacteria, probiotic.

INTRODUCTION

Functional foods are a trend for industry for healthy eating and consumers' interest as a new alternative to prevent diseases such as allergies, intestinal problems and cardiovascular diseases among others (Ahmed et al., 2013; Altay et al., 2013). These functional foods are based on microorganisms' use that affect consumers' health positively, in the form of fermentation process and/or in the use of microbial biomass (Corona et al., 2016).

The functional food microorganisms include lactic acid

bacteria such as *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Staphylococcus* (Duarte et al., 2010; Magalhães et al., 2010) for fermented foods of plant or animal origin and *Saccharomyces* yeasts for alcohol production (Dhaliwal et al., 2011). Kefir grains has also been used for fermentation of milk and fruits (Magalhães et al., 2010; Nalbantoglu et al., 2014). Kefir is a beverage prepared by kefir grains fermentation in different substrates and is a combination of yeasts and bacteria's in a symbiotic polysaccharide matrix (Puerari et al., 2012;

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Table 1. Fermented foods produced by lactic acid bacteria and *Saccharomyces cerevisiae*.

Microorganisms	Fermented products	References
Lactic acid bacteria		
<i>Streptococcus thermophilus</i> , <i>Lactobacillus bulgaricus</i>	Yogurt	Ahmed et al., 2013; Altay et al., 2013
<i>Lactobacillus casei</i> , <i>Bifidobacterium animalis</i>	Fermented milk	Corona et al., 2016
<i>Lactococcus</i> sp., <i>Leuconostoc</i> sp.	Fermented milk	Magalhães et al., 2010; Nalbantoglu et al., 2014
<i>Lactobacillus</i> sp.	walnut milk beverage	Cui et al., 2013
<i>Lactobacillus</i> sp., <i>Lactococcus</i> sp., <i>Leuconostoc</i> sp.	Cheese whey beverage	Magalhães et al., 2010
Yeast		
<i>Saccharomyces cerevisiae</i>	Fruits beverages	Dias et al., 2007; Dhaliwal et al., 2011; Andrade et al., 2017
<i>Saccharomyces cerevisiae</i>	Cocoa pulp-based beverages	Puerari et al., 2012
<i>Saccharomyces cerevisiae</i>	Vinegar	Viana et al., 2017

Guedes et al., 2014). The microorganisms present in kefir grains are non-pathogenic bacteria and yeasts (Magalhães et al., 2010; Nalbantoglu et al., 2014).

In general, fermentation process has been shown to preserve food with modification of physicochemical properties of foods but also provide impact on the raw material functional quality (Ahmed et al., 2013; Altay et al., 2013). Microorganisms are also used for the production of enzymes, pigments, vitamins, polysaccharides and prebiotics (non-digestible ingredients that benefits the host health by stimulating the bacteria in the gut) (Magalhães et al., 2010; Rodrigues et al., 2016).

Microalgae have also been used in the functional foods supplementation (Mata et al., 2010; Hoseini et al., 2013; Vaz et al., 2016). Microalgae are generally used in the lyophilized biomass form. *Spirulina* microalgae are the most used in food supplementation (Hoseini et al., 2013; Vaz et al., 2016). Nutritional bioactive compounds produced by microalgae's can meet the energy needs of the consumer and prevent chronic disease (Christaki et al., 2011; Hoseini et al., 2013; Vaz et al., 2016).

This review reports detail of the use of microorganisms for functional food supplements in the form of fermentation of the substrate and/or addition of lyophilized microbial biomass. The data from this review can be useful to support the development of new functional products for the market.

Lactic acid bacteria

A crucial question for the manufacture of fortified fermented food by lactic acid bacteria: is increased nutraceutical compounds formed by these microorganisms? (Corona et al., 2016). Lactic acid bacteria produce various products, such as dairy beverage, fermented milk and yogurt (Table 1). Lactic acid bacteria, *Streptococcus thermophilus* and *Lactobacillus bulgaricus* produce yogurt (Ahmed et al., 2013; Altay et al., 2013). The fermented milk is also produced by other lactic acid bacteria such as *Lactobacillus casei* and *Bifidobacterium*

animalis. The fermented milk products have been produced since around 10,000 BC (Corona et al., 2016).

Lactic acid bacteria's are industrially important all over the world in industrial food fermentations (Ahmed et al., 2013; Altay et al., 2013). Their contribution primarily consists of formation of lactic acid from the lactic fermentation resulting in an acidification of the formed products, which is a factor in the preservation of these products. Lactic acid bacteria also have the ability to contribute to flavour, texture and formed product nutrition (Ahmed et al., 2013; Altay et al., 2013). Lactic acid bacteria are ideal for the production of important nutraceuticals in fermented foods. In this review, the nutraceuticals produced by these microorganisms as prebiotics (vitamins, low-calorie sugars) and components that improve the health of consumers, through growth stimulation of probiotics were described (Table 2) (Abd-Rabou et al., 2010).

Low-calorie sugars body-weight control is a concern in all countries and a new food products elaboration containing low-calorie sugars is required (Ahmed et al., 2013; Altay et al., 2013). Sorbitol and mannitol are low-calorie sugars that replace sucrose, glucose, fructose, lactose and glucose in food (Ahmed et al., 2013; Corona et al., 2016). Mannitol also serves as antioxidant for consumers (Corona et al., 2016).

Heterofermentative lactic acid bacteria are known for their ability to produce mannitol in fermentative process (Marsh et al., 2013). Production of sorbitol can be induced in these bacteria in fructose addition in fermentation substrate (Ahmed et al., 2013).

Tagatose is another carbohydrate that is considered as sucrose replacement. It has much lower caloric value because the human body poorly degrades it. It has recently been launched as lower caloric sugar (Marsh et al., 2013). Tagatose is formed in lactose degradation by lactic acid bacteria (Marsh et al., 2013).

Trehalose is another low-calorie sugar. It is partially digested by humans, and is considered a dietetic sugar. It is metabolised by lactic acid bacteria such as *S. mutans* and *S. salivarius* (Corona et al., 2016). Other biological

Table 2. Nutraceuticals produced by lactic acid bacteria.

Nutraceuticals	References
Sorbitol	Ahmed et al., 2013; Corona et al., 2016
Mannitol	Ahmed et al., 2013; Marsh et al., 2013; Corona et al., 2016
Tagatose	Marsh et al., 2013
Trehalose	Marsh et al., 2013
Folate (vitamin B11)	Marsh et al., 2013; Corona et al., 2016
Cyanocobalamine (vitamin B12)	Marsh et al., 2013; Corona et al., 2016; Abd-Rabou et al., 2010

activities than is attributed to trehalose are different stress conditions decrease (Marsh et al., 2013).

Lactic acid bacteria produce vitamins such as folate (vitamin B11) and cyanocobalamine (vitamin B12) (Marsh et al., 2013; Corona et al., 2016).

Folate or folic acid (chemically synthesized vitamin) is an essential vitamin for human diet. It is involved in the biosynthesis of deoxyribonucleic acid (DNA) and Ribonucleic Acid (RNA). For pregnant women, folic acid is recommended to prevent neural-tube defect in newborns (Corona et al., 2016). It is even reported against some forms of cancer (Marsh et al., 2013; Corona et al., 2016). Folic acid is produced by lactic acid bacteria (Marsh et al., 2013).

Some lactic acid bacteria have long been known for their production capacity of vitamin B12. These microorganisms produce coenzyme B12 or deoxyadenosylcobalamin via a pathway involving the starting precursor's uroporphyrinogen III (precursor for heme, F430 and cobalamin), dimethylbenzimidazole and adenosyl-moiety. Many fermentative processes have been described for the production of vitamin B12 from lactic acid bacteria varying the nutrient composition of the substrate, such as amino acid composition including cobalt ions (Abd-Rabou et al., 2010).

Examples have been presented where significant production of nutraceuticals fermented products can be induced by lactic acid bacteria. These microorganisms, undoubtedly, is a new way of supplementation functional fermented foods.

***Saccharomyces cerevisiae* yeast**

S. cerevisiae is the most thoroughly known eukaryotic microorganism, which aids our understanding of biology. This yeast has been used in the production of alcoholic beverages, and today this microorganism is used in functional food industries processes (Dias et al., 2007; Dhaliwal et al., 2011; Andrade et al., 2017). *S. cerevisiae* is a non-pathogenic microorganism, and due to its long application in the production of consumable products, it has been classified as a generally regarded as safe (GRAS) organism (Dhaliwal et al., 2011). In addition, the well-established fermentation technology for large-

scale production with *S. cerevisiae* makes this organism attractive for several biotechnological purposes, as the production of fruits and vegetables beverages containing nutritional values (Table 1).

The substrates metabolized by *S. cerevisiae* serves as an important option in utilization of new raw materials, e.g., a variety of fruits and vegetables (Dias et al., 2007; Dhaliwal et al., 2011). The utilization of molasses (glucose, fructose, sucrose and trisaccharide raffinose) expands the range of substrates used by *S. cerevisiae*. Molasses, which is used for ethanol and fermented beverages production, contains mainly glucose, fructose, sucrose and disaccharide raffinose (Dias et al., 2007; Dhaliwal et al., 2011; Andrade et al., 2017).

Several compounds are produced by *S. cerevisiae* in fermentation, including alcohols, ethyl esters, acetates, monoterpene alcohols, volatile fatty acids and aldehydes, providing pleasant and nutritional characteristics of fermented beverages (Dias et al., 2007; Dhaliwal et al., 2011; Andrade et al., 2017). Propionic and citric acid is metabolized by *S. cerevisiae*. Propionic acid is an important odor-active compound in fruits and vegetables pulp, and citric and malic acids are in fruit and vegetables fermented beverages, acting as preservatives with antimicrobial properties (Dias et al., 2007; Duarte et al., 2010). The organic acids produced by *S. cerevisiae* species, contribute to the refreshing flavour and fermented beverages aroma (Duarte et al., 2010). The alcohols produced by *S. cerevisiae* provide pleasant taste in beverages besides presenting role in the conservation of beverages (Dias et al., 2007; Duarte et al., 2010; Dhaliwal et al., 2011).

Fruits and vegetables pulp-based fermentation by *S. cerevisiae* can produce novel beverages of acceptable organoleptic character. This is significant because new processing fruits can be used to minimize production losses, to generate more profits and introduce new products of nutritional value to the market.

KEFIR

Kefir is fermented milk beverage, widely known as an excellent source of probiotics with potential health benefits (Magalhães et al., 2010; Nalbantoglu et al.,

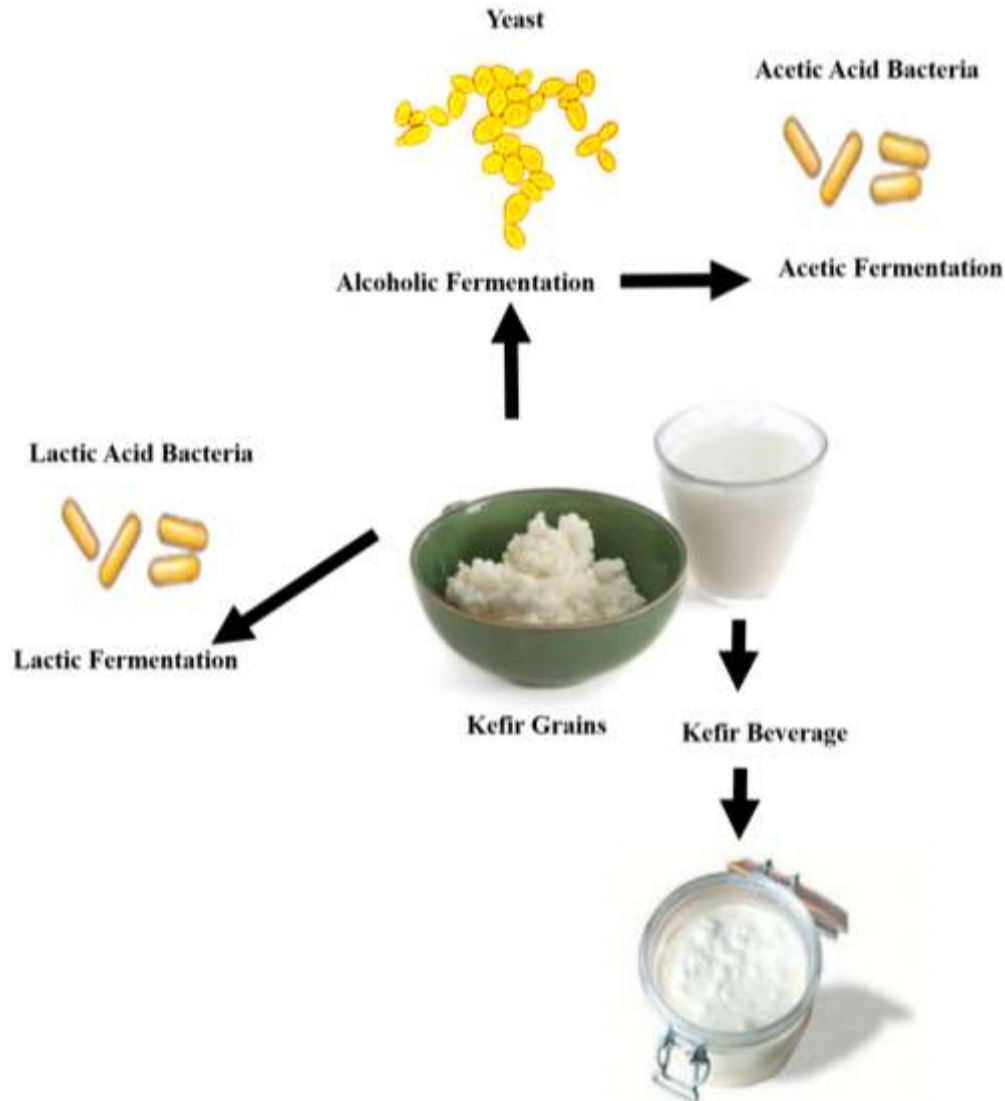


Figure 1. Microbial interaction model proposed for milk kefir.

2014). Kefir beverage is a traditional to Middle Eastern. It originated in the Caucasus in Asia thousands of years ago (Magalhães et al., 2010; Nalbantoglu et al., 2014). Kefir is a symbiosis between yeast and bacteria (Figure 1). A vast variety of different species of microorganisms forms the kefir grains (Magalhães et al., 2010; Nalbantoglu et al., 2014). The *Lactobacillus* genera are the most frequent in kefir. Other lactic bacteria including *Lactococcus* and *Leuconostoc* genera are also common in kefir (Magalhães et al., 2010; Nalbantoglu et al., 2014). *Acetobacter* genera represent acetic bacteria and the yeast isolates are *Kluyveromyces*, *Candida* and *Saccharomyces* genera (Leite et al., 2013; Marsh et al., 2013).

The microbial species from kefir grains carry out three types of fermentation during the process: lactic, alcoholic and acetic. The kefir grains can easily adapt to different

substrates and lead to production of new probiotic products (Magalhães et al., 2010; Nalbantoglu et al., 2014). Recent studies show new functional kefir beverages such as whey-based beverages (Magalhães et al., 2010), cocoa pulp-based beverages (Puerari et al., 2012), walnut milk beverage (Cui et al., 2013) and vinegar kefir (Viana et al., 2017).

“Traditional” way of kefir beverage production is using pasteurized or Ultra-high-temperature processing (UHT) treated milk (Figure 2). The milk is mixed with the kefir grains. The mixture is left to stand at room temperature for approximately 24 to 48 h. The fermented kefir is filtered to obtain the kefir grains. This kefir beverage is appropriate for consumption. Kefir grains are added to new milk, and the process is repeated.

Kefir beverage is mainly considered a probiotic resource (Leite et al., 2013). Kefir may help bridge the

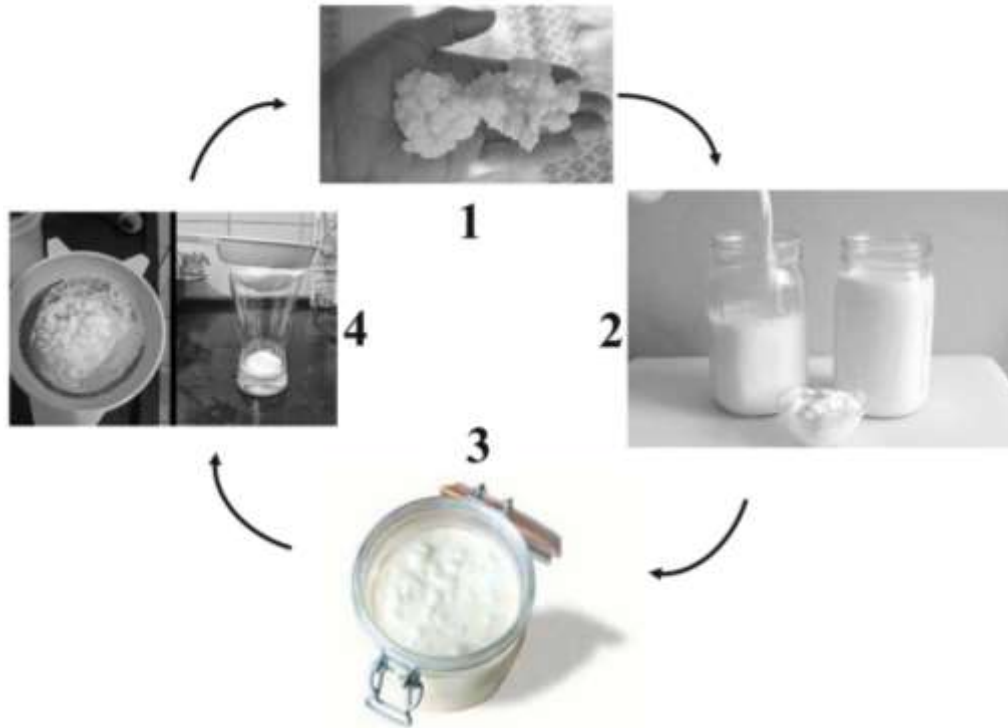


Figure 2. Milk kefir beverage production. Kefir grains (1) are added to milk and are left to stand at room temperature for fermentation for 18-24 h; (2) the milk is then fermented forming the kefir beverage (3) after which they are filtered (4) and ready to start another cycle. The fermented milk that results from step 3 is appropriate for consumption.

gap between the health benefits and consumption of non-dairy foods and provide the benefits of probiotic without milk (Leite et al., 2013; Garofalo et al., 2015). Kefir can ferment non-dairy substrates such as fruits, cereals and vegetables (Magalhães et al., 2010; Leite et al., 2013; Nalbantoglu et al., 2014; Garofalo et al., 2015).

The microorganisms in the kefir grains produce lactic acid and antibiotics, which inhibit the development of pathogenic microorganisms in kefir (Leite et al., 2013; Garofalo et al., 2015). Furthermore, kefir beverage ingestion provides decreased levels of *Enterbacteriaceae* in the intestinal tract (De Oliveira Leite et al., 2013). Other important bioactivities have been tested in kefir beverage. Anti-inflammatory effect (Diniz et al., 2003), anti-ulcerogenic (Rodrigues et al., 2016), antioxidant (Alsayadi et al., 2013), cicatrizing (Moreira et al., 2008), antimicrobial (Fiorda et al., 2016) and healing activity activities (Rodrigues et al., 2016) have been reported.

Recently, Kefir grains fermented in honey produced a kefir beverage with health characteristics, such as antioxidant capacity and DNA protection effect (Fiorda et al., 2016). Kefir contributes to defence system against oxidative damage reactions, avoiding formation of free radicals and/or repairing the damage caused by them (Fiorda et al., 2016).

Due to the numerous positive effects of kefir on the

human health, alternative substrates may be used for kefir grains fermentation. Fruits, molasses or vegetable are used as medium of fermentation for kefir grains (Magalhães et al., 2010; Nalbantoglu et al., 2014). Fruits are the most diverse group of alternative substrates for kefir fermentation. Typical fruits, such as apple, strawberry, pear and grape, as well as region-specific fruits (pomegranate and quince) can be used. This allows for production of new functional and probiotic beverages, with characteristics similar to traditional kefir beverage (Grønnevik et al., 2011).

The adaptation of kefir grains into different substrates has shown potential for production of kefir beverages with distinct sensory characteristics and functional proprieties.

***Spirulina* microalgae biomass**

Applications of microalgae biomass for food supplementation have developed significantly over the last years (Mata et al., 2010; Hoseini et al., 2013; Vaz et al., 2016). *Spirulina* microalgae biomass is important for consumer providing several biological benefits, such as anti-inflammatory, antioxidant, anti-obesity and anti-carcinogen (Guedes et al., 2011; Pangestuti and Kim, 2011). *Spirulina* biomass supplements various products.



Figure 3. Supplemented foods with *Spirulina* microalgae.

These microalgae are integrated into the nutritional formula of cheese, cookie, cake and fruits beverages (Vilchez et al., 2011) (Figure 3).

Spirulina biomass (60% dry weight) protein percentage exhibits high digestibility and contains all essential amino acids (Vaz et al., 2016). *Spirulina* biomass is protein sources rich (Guedes et al., 2011; Pangestuti and Kim, 2011; Vaz et al., 2016). *Spirulina* microalgae biomasses are also sources of antioxidant and vitamins. These microorganisms are a source ascorbic acid, B1, B2, B3,

B6, B9, B12 vitamins, folic acid and biotin (Christaki et al., 2011). *Spirulina* is also rich in vitamin B12 and β -carotene and their consumption facilitates the vitamin B1 absorption from foods (Vaz et al., 2016).

Spirulina microalgae pigments also are important for consumer providing health. The major pigment in microalgae is the carotenoids. Currently, carotenoids are mainly used for fortified foods and food dyes (Guedes et al., 2011; Pangestuti and Kim, 2011; Vaz et al., 2016). Chlorophyll, present in *Spirulina* microalgae, is also used

as a nutritional source (Vaz et al., 2016). In addition to chlorophyll, *Spirulina* microalgae biomass contains phycocyanin, which is an antioxidant blue pigment (Guedes et al., 2011; Pangestuti and Kim, 2011; Vaz et al., 2016).

Rabelo et al. (2013) found that adding *Spirulina* microalgae increased the nutritional quality of the produced foods. The authors observed an increase in the contents protein, lipids, fibers and minerals. Figueira et al. (2011) developed gluten-free bread for consumers with celiac syndrome. The authors found that the breads produced supplemented with *Spirulina* microalgae biomass showed higher protein content and better amino acid composition when compared with the control (gluten-free bread without *Spirulina* microalgae).

Therefore, *Spirulina* microalgae biomass is a promising food source. These microalgae are active ingredients source for functional food supplements. Thereby, new functional foods supplemented with *Spirulina* microalgae biomass promote market future prospects.

Considerations

The importance of foods supplementation with microorganisms in food industry is a good alternative of further research. Scientific study should be performed on the relations between different microorganisms and human consumers and how these relations can result in nutritional and therapeutic benefits, as curing and/or preventing human diseases.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Full Length Research Paper

Antimicrobial activity of some lactic acid bacteria isolated from local environment in Egypt

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The aims of this study were to investigate antimicrobial activities of what so-called wild strains of LAB isolated from different sources of Egyptian environment including raw and fermented milk, cheeses, other dairy products, sausage and silage. Isolation of lactobacilli and lactococci were carried out onto plates of MRS and M17 agar, respectively, grouped to identification and the obtained treated supernatants were tested for their antimicrobial activities against the indicators bacteria. The proteinaceous nature of each cell-free supernatant fluid was evaluated by heat sensitivity test as well as treating with some proteolytic enzymes. Furthermore, the antimicrobial activity, of seventy-eight isolates of LAB against the reference strains, was determined by the agar diffusion method. Some environmental LAB isolated in Egypt are capable of synthesizing inhibitive substance(s) against some pathogenic/spoilage bacteria being these inhibitive substances, a proteinaceous in nature which act differently against the tested indicators bacteria.

Key words: Antimicrobial activity, Egyptian environment, food, LAB, food safety.

INTRODUCTION

Antimicrobial agents are of enormous value for combating infectious diseases. Their efficacy has been threatened by possible spread of the phenomena of the "microbial resistance" since many microorganisms start to gain resistance to the antibiotics (Teuber et al., 1999). Searching for natural alternatives to the antibiotics is now

a goal of many researchers. They have been tried not to use in treating gastrointestinal disease only, but also to use in food industry as natural antimicrobial substances that many have a bacteriostatic or bacteriocidal effect against many food borne pathogens (Soomro et al., 2002; Oyetyayo et al., 2003; Savadogo et al., 2004).

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Immune modulation by probiotics was recognized as an important aspect of host gut health (Papadimitriou et al., 2015). It has been suggested that the health benefits of some probiotics used in functional foods and pharmaceutical preparations are due to the capacity of these microorganisms to stimulate the host immune system (Perdigon et al., 2002).

Lactic acid bacteria (LAB), which is naturally occurred in our environment, may act as source for such natural antimicrobial compounds. This antimicrobial effect of LAB has been appreciated by man for more than 1000 years ago and has enabled him to extend the shelf life of many foods through fermentation processes since it can inhibit the growth of such microorganisms including spoilage and pathogenic bacteria (Okereke et al., 2012; Aslim et al., 2005). This effect may have resulted from the action of organic acids, hydrogen peroxide, diacetyl and/or a compound(s) called bacteriocin(s) (Gonzalez et al., 2010; Oyetayo et al., 2003; Savadogo et al., 2004). Bacteriocins are low molecular-mass peptides or protein with a bacteriostatic or bacteriocidal mode of action in particular against closely related species (Deraz et al., 2005). These bacteriocins may contribute to the competitiveness of the producing strain in the environmental product ecosystem (Caplice and Fitzgerald, 1999). An arid variety of LAB, that produce antimicrobial substances such as hydrogen peroxide, CO₂, diacetyl, acetaldehyde, D-isomers of amino acids, reuterin and bacteriocins have already been isolated from many fermented products. This includes naturally fermented sausages (Aslim et al., 2005), fermented milk and dairy products (Morea et al., 1999) and silage which is stored fermented vegetable product (Ennahar et al., 1996).

The aims of this study were to screen the antimicrobial activities of what so-called wild strains of LAB isolated from different sources of our environment including raw and fermented milk, cheeses, other dairy products, sausage and silage. Thus, the potential of these isolates by new antagonistic metabolites, other than acids and hydrogen peroxide, to inhibit food borne pathogens as well as food spoilage bacteria was evaluated.

MATERIALS AND METHODS

Samples

In this study, fifty-two samples were collected from different locations in Cairo and Giza governorates. The collected samples included four samples each of raw sheep, goat and buffalo's milk, five samples of yoghurt (*zabady*), four samples of naturally acidified milk (*Rayeb*), five samples each of *Kareish* and *Domiat* cheese (soft type), seven samples of *Ras* cheese (hard type), five samples of ice cream, four samples of naturally fermented sausage, two samples of naturally fermented silage as well as three samples of fresh plants. Samples were immediately placed in ice box and transferred to the Laboratory where they were subjected to the microbiological analysis in the day of purchasing. In exceptional instances, the samples were stored at refrigerator temperature for up to a maximum of 24 h before analysis.

Cultures and media

The indicator organism strains were obtained from the stock cultures of the Agricultural Research Centre in Giza including *Escherichia coli* 0157: H7 ATCC 6933, *Bacillus cereus* ATCC 33018, *Staphylococcus aureus* ATCC 20231, *Salmonella typhimurium* ATCC 14028, and *Pseudomonas aeruginosa* ATCC 9027. *Listeria monocytogenes* V7 strain serotype 1 (milk isolate), was obtained from the Department of Food Science from University of Wisconsin-Madison (USA). All strains were routinely maintained by sub culturing once a week in tryptone soya broth/agar and stored at 4°C until use. The purity of all strains was checked before each use. All media were purchased from Oxoid (Madrid, Spain). MRS agar/broth was used for isolation/cultivation of lactobacilli from the examined samples; however M17 agar/broth were used for isolation/cultivation of lactococci. Tryptone soya broth was used for cultivation and propagation of all the tested bacterial strains. Preservation and propagation of all species of LAB was done using litmus milk.

Isolation

Twenty-five grams of each sample was aseptically transferred to plastic bag and homogenized in 225 ml of sterile buffered peptone water (BPW). Ten-fold dilutions of homogenates were prepared and inoculated onto plates of MRS and M17 agar for isolation of lactobacilli and lactococci, respectively. MRS plates were incubated in anaerobic condition using CO₂ generated kits (Anaerobe-Gen, Oxoid) and incubated at 37°C, while M17 plates were incubated in normal aerobic conditions at 32°C. All plates were incubated for a period of three to five days. Characteristic colonies of LAB were counted and expressed as colony forming units (cfu/g or ml). Three to five of typical isolates from each sample were picked up, grown, purified and maintained on MRS slants for future grouping.

Grouping

The isolates of LAB were propagated twice in MRS broth at 37°C. Then inoculums of 16 hours old grown onto MRS agar were mainly identified, according to Bergey's Manual (2009), for their morphological characteristics, including the cell form and their cellular arrangement, Gram-stain reaction (Brock et al., 1994), catalase production (Herrero et al., 1996) as well as their ability to produce gas from glucose (Kandler and Weiss, 1986). The grouped isolates were then kept in litmus milk, supplemented with 1% glycerol, for future identification.

Culture supernatant

The isolates of LAB were propagated overnight (16 h) in 100 ml MRS broth (pH 6.8). A cell free solution was obtained by centrifugation of culture at 5000 rpm at 4°C for 20 min, followed by filtration of supernatant through sterilized filter (0.2 µm, Millipore). To avoid the effect of the produced organic acids, the obtained cell-free supernatant was neutralized to 6.5-7.0 using 1 N sodium hydroxide. Also, the effect of hydrogen peroxide was eliminated by the addition of 5 mg. Catalase per each one ml of the supernatant and left at 25°C for 30 min. The obtained treated supernatants were tested for their antimicrobial activities against the indicator bacteria.

Proteinaceous nature treatments

The proteinaceous nature of each cell-free supernatant fluid was

Table 1. Maximum and minimum counts of LAB/g using MRS and M17 agar plates.

Sample	MRS				M17				Total MRS and M17	Total No.
	Max.	Min.	No. of isolates	No. ^a	Max.	Min.	No. isolates	No. ^a		
Silage (2)	4.6 ×10 ⁵	4.2 ×10 ³	8	5	2.3 ×10 ⁴	3.0 ×10 ³	7	2	15	7
Ras cheese (7)	1.4 ×10 ⁸	1.0 ×10 ⁷	30	8	1.8 ×10 ⁸	1.7 ×10 ⁷	21	6	51	14
Sausages (4)	2.5 ×10 ⁵	4.2 ×10 ³	20	5	1.6 ×10 ⁵	1.7 ×10 ³	10	2	30	7
Raib Milk (4)	2.3 ×10 ⁹	4.7 ×10 ⁸	16	7	2.0 ×10 ⁹	3.4 ×10 ⁷	11	2	27	9
Yoghurt (5)	4.4 ×10 ⁹	2.8 ×10 ⁷	22	9	5.3 ×10 ⁹	5.4 ×10 ⁷	14	0	36	9
Domiat cheese (5)	2.7 ×10 ⁸	3.7 ×10 ⁵	24	7	7.2 ×10 ⁸	2.3 ×10 ⁶	15	2	39	9
Goat Milk (4)	2.3 ×10 ⁹	6.6 ×10 ⁷	18	5	2.2 ×10 ⁹	5.2 ×10 ⁷	12	0	30	5
Buffalo Milk (4)	3.2 ×10 ⁸	2.3 ×10 ⁷	19	5	2.3 ×10 ⁸	1.4 ×10 ⁷	12	2	31	7
Sheep Milk (4)	4.4 ×10 ⁸	4.3 ×10 ⁷	18	4	2.5 ×10 ⁸	3.3 ×10 ⁷	13	1	31	5
Karish cheese (5)	6.2 ×10 ⁸	7 ×10 ⁶	21	4	4.7 ×10 ⁸	3 ×10 ⁶	12	2	33	6
Surface Green Plants (3)	3.6 ×10 ⁶	3.3 ×10 ⁴	10	0	2.2 ×10 ⁶	2.2 ×10 ⁴	0	0	10	0
Ice Cream (5)	3.2 ×10 ⁴	4.5 ×10 ³	14	0	6.3 ×10 ⁴	2.5 ×10 ³	0	0	14	0
			220	59			127	19	347	78

Number between brackets is the numbers of the analyzed samples. ^aNo. of isolates that produce proteinaceous inhibitive substance against one or more of the tested indicator organisms.

evaluated by heat sensitivity test as well as treating with some proteolytic enzymes. Supernatants were heated for 10 min at 85°C and the loose in their activities was evaluated by the agar diffusion assay method. The same was true after treating the supernatant with various proteolytic enzymes including trypsin and α-chymotrypsin. In this case each enzyme was dissolved in sterile 4 ml phosphate buffer pH 7 and added to the cell free culture supernatants fluid to obtain a final concentration of 1 mg/ml (Bonade et al., 2001). Control samples contained only buffer solution were done. The samples with and without enzymes were incubated for one hour at 37°C.

Detection of the antimicrobial activity

The antimicrobial activity, of seventy-eight isolates of LAB against the reference strains, was determined by the agar diffusion method (Settanni and Corsetti, 2008). One ml culture of the activated indicator strain(s) (10⁴ to 10⁵ cell/ml) was inoculated into 20 ml of Mueller-Hinton agar (Becton Dickinson, USA) and poured in Petri dishes. After solidification of the agar, wells of 5 mm diameter were cut in, and 50 µm of the treated supernatant fluid, from a culture of strain under test, was added to each well. Dishes were stored for 2 h in refrigerator followed by incubation for 24 h at 37°C. The diameters of the inhibition zones were measured (Rammelsberg and Radler, 1990).

RESULTS

A total of fifty-two samples of food or feed products were investigated for their load of LAB. Maximum and minimum counts obtained on media of MRS and M17 agar plates are given in Table 1 which demonstrated that high LAB population ranged from 10⁹ to 10⁷ densities were detected in fermented milk products including Rayeb and yoghurt milk followed by raw goat's milk and cheeses (Ras, Domiat and Karish) and ended by buffalo and sheep raw milk. Samples of feed products reported

populations ranged from 10⁶ to 10³ at surface of green-plants and silage however the lowest counts of LAB ranged from 10⁵ to 10³ were detected in sausage and ice-cream. The bacteriostatic or bacteriocidal effect of cell-free extracts of LAB that either picked up from MRS or M17 plates, against the tested pathogenic microorganisms expressed by the detected clear zones of inhibition, are listed in Table 1. In our study, 347 LAB were isolated from food and feed products and picked from both the used types of media, were found to have this action of inhibition. In this case, their effect was based on the action of metabolites of LAB (bacteriocins proteinaceous synthesizing substances produced by such strain, as well as a combination of antimicrobial substances such as hydrogen peroxide and organic acids). After elimination of the effect of acidity and hydrogen peroxide of these extracts, the number of the effective extracts / isolates was reduced to 78. The source from which these isolates have been isolated is shown in Table 1. Of these isolates, 19 strains were spherical however 59 were rods/coco bacilli in shape or in their cell form. The lowest counts of LAB detected in ice-cream samples could be explained by the fact that the ice-cream mixtures have been previously exposed to high temperatures, during the pasteurization process of the product. Results in our study indicate that, all the selected 347 isolates of LAB were Gram-positive and catalase-negative, 127 isolates were spherical however, and the other 220 isolates were rods/coco bacilli in shape. All the isolates were not able to produce gas from glucose. The only exception was two strains, rods, gave non-significant amount of gas.

The source of these isolates as well as their efficacy and spectrum of action based on the action of bacteriocins like substances, expressed as diameters of

inhibition against the indicator microorganisms are shown in Table 2. A wide range of the reported inhibition zones ranged from 6 to 12 mm, for all the tested 78 LAB isolates, against the various tested indicators was obtained. The biggest diameter (12 mm) was obtained using the extract of isolates S33 which have been isolated from Domiati cheese, against the indicator *P. aeruginosa* however, the smallest diameter (6 mm) was obtained with the extracts of many isolates originated from all the investigated products against all the different tested pathogenic indicators. According to the Table 2, bacteriocins produced by LAB isolates were effective against Gram-negative indicators than Gram-positive. Where, out of the 78 bacteriocin cell-free extracts, S 52, S 33 and S 13 extracts were effective against Gram-negative indicators including *E. coli* 0157:H7, *S. typhimurium* and *P. aeruginosa* respectively, while only S 32, S 75 and S 7 extracts were effective against *S. aureus*, *L. monocytogenes* V7 and *B. cereus*, respectively. This phenomena may depend on the species of the bacterial indicators used in this work and also may provide insight into other mechanisms that may happen in the bacterial cell wall and contribute to these antimicrobial effects. Other explanation could be the functional capacities of the bacteriocin itself which could be explained by specific- specific mechanisms of action. Although the numbers of bacteriocins, produced by LAB isolates, effective against Gram-negative indicators were more than that those effective against Gram-positive, but it is found that when the individual extract was effective in the same time against both Gram-positive and Gram-negative, the Gram-positive bacteria were more susceptible to the bacteriocin than did Gram-negative. For example as is shown in Table 2, the extract of strain S3, isolated from sausage, gave inhibition zone of 7 mm against *S. typhimurium*, however it gave 10, 9 and 8 mm inhibition zone diameter against *B. cereus*, *S. aureus* and *L. monocytogenes*, respectively.

The bacteriocin-producing (bacteriocin-like substances) strains (78 isolates) were subjected to identification according to their morphological, cultural, physiological and biochemical characteristics as described in Bergey's Manual of Systematic Bacteriology, 2009. Of the identified 78 LAB isolates, 19 isolates were found to belong to genus *Lactococcus* however the other 59 isolates were referred to genus *Lactobacillus*. The representative species of the total cocci were identified as 3 strains *Lactococcus lactis* sub sp. *Lactis* and 16 strains *Streptococcus thermophilus*. On the other hand, the identified strains belonging to genus *Lactobacillus* were 13 strains of *Lactobacillus jensenii*, 12 strains *Lactobacillus acidophilus*, 7 strains *Lactobacillus casei* sub sp. *rhamnosus*, 6 strains *Lactobacillus plantarum*, 6 strains *Lactobacillus helveticus*, 6 strains *Lactobacillus gasserii*, 5 strains *Lactobacillus reuteri*, 3 strains *Lactobacillus delbrueckii* sub sp. *bulgaricus* and one strain of *L. casei* sub sp. *casei* (Table 3).

DISCUSSION

The presence of high counts of LAB detected in cheese and fermented milk possibly due to the natural fermentation processes and/or the addition of lactic starters during the processing of such products (Ayad et al., 2004; Tamine and Robinson, 2007). Fluctuated numbers of LAB detected in different types of the investigated raw milk may be depended on the methods used in handling and storage of this product as well as the hygienic status of the animal that produced such milk (Ayad et al., 2004). The environmental stress may explain the moderate counts of LAB, detected in the feed samples including surface of green plants (Ranong, 1999). These low LAB counts found in sausage samples may be due to the use of sausage spices and preservatives that might be added during processing (Giuseppe et al., 2005). The antimicrobial effect of these 78 isolates has not been changed after heating at 85°C for 10 min; however this effect completely inactivated after treatment with the proteolytic enzymes indicating that these inhibitory compounds, produced by such isolates, are proteinaceous nature which consider the main characteristic of any bacteriocin (Herrerros et al., 2005).

According to our Table 2, similar results have been reported by many investigators in different countries (Savadogo et al., 2004). The extent by which Gram-positive and Gram-negative indicator organisms were susceptible to the bacteriocin-like substances is presented in Table 2. Savadogo et al. (2004) reported that action of the bacteriocins may explain by the interaction with lipoteichoic acids, which are absent in Gram-negative bacteria, and these molecules would play the role of sites needed to produce the bacteriocinal effect. This last observation is in accordance of other reports and studies which stated that some bacteriocins produced by Gram-positive bacteria, regardless of its source, have a broad spectrum of activity against Gram-positive microorganisms than did with Gram-negative (Savadogo et al., 2004). Others reported that bacteriocins from normal inhabitant of gastrointestinal tract of humans and animals have antagonistic actions against wide range of Gram-positive and in some instances for also Gram-negative bacteria. Lima and Filho (2005) found that 265 out of 474 LAB produced bacteriocins which demonstrated activity against many Gram-positive organisms however the only Gram-negative susceptible organism was *Salmonella* spp. We believe that further understanding of the molecular foundation of the relationship between microbes (bacteriocin-producer)-microbes (inhibited-organism) should/may explain or at least clarify these findings.

Isolation of such LAB species from foods was reported by many investigators in different countries. In Morocco Benkerroum et al. (2000) isolated a bacteriocin-producing *L. lactis* sub sp. *Lactis* strain from a soft white cheese

Table 2. Inhibition of various indicator organisms by bacteriocin-like substances produced by isolated LAB.

Product	Diameter of inhibition (mm)	Inhibited indicator organism(s)	LAB
Silage	8	Sal	S1
	7,9	Sal, Pse	S21
	9,10	Li., Sta	S27
	10	Sal	S43
	7,8	Sal, Bc	S58
	6	Ec	S67
	6	Sal	S73
Ras cheese	6,8	Ec, Sta	S2
	6	Sal	S4
	7,9	Bc, Sta	S7
	8	Sal	S11
	7	Sal	S17
	9	Sal	S24
	6,10	Pse, Bc	S34
	6,6	Pse, Ec	S38
	7	Sal	S46
	8	Pse	S61
	6	Sal	S71
	7	Sal	S74
	8	Li	S77
7,9	Pse, Li	S78	
Sausage	7,10,9,8	Sal, Bc, Sta, Li	S3
	9	Sta	S10
	8	Ec	S20
	7	Li	S41
	6,6,7,8	Sal, Pse, Ec, Sta	S47
	8,6	Sal, Pse	S59
	7	Sta	S68
Rayeb	9	Pse	S59
	7,8	Pse, Bc	S9
	7	Sal	S19
	8	Sta	S26
	6,10	Sal, Pse	S36
	8	Ec	S40
	7	Sal	S45
	7,6,8	Sal, Pse, Bc	S60
6	Li	S69	
Yoghourt	8	Ec	S6
	6	Li	S18
	7	Sta	S25
	11	Pse	S35
	7	Pse	S39
	8,6	Bc, Ec	S51
	7,6,8	Sal, Pse, Ec	S52
	6,8	Pse, Sta	S64
7	Pse	S70	

Table 2. Contd.

Domiaty cheese	7,8	Pse, Bc	S8
	10	Sal	S12
	10,6	Sal, Pse	S15
	6,7,7,8	Sal, Ec, Pse, Li	S16
	6,7,8	Sal, Pse, Li	S23
	6,8,10	Pse, Sta, Li	S31
	9,12	Sal, Pse	S33
	9,9	Sal, Pse	S62
	7,9	Sal, Bc	S76
Raw goat milk	7,10,6	Sal, Pse, Ec	S13
	6	Li	S30
	9,6	Bc, Ec	S50
	8,6	Sal, Pse	S53
	6	Pse	S65
Raw buffalo milk	6,9,10	Sal, Pse, Bc	S14
	6,8	Sal, Ec	S49
	6	Pse	S29
	9	Sal	S55
	8	Sal	S56
	8,10	Sal, Pse	S66
	9,10	Li, Sta	S75
Raw sheep milk	7,9,8	Sal, Sta, Li	S22
	6,9	Sta, Li	S32
	7,8	Pse, Sta	S48
	7	Sal	S54
	11	Pse	S63
Karish cheese	10	Bc	S28
	6,6,10	Sal, Pse, Sta	S44
	9,6	Sal, Pse	S37
	6	Li	S42
	8	Sal	S57
	6	Sal	S72

Bc = *Bacillus cereus*; Ec = *Escherichia coli* O157:H7; Li = *Listeria monocytogenes*; Pse = *Pseudomonas aeruginosa*; Sal= *Salmonella typhimurium*; Sta= *Staphylococcus aureus*.

effective against *L. monocytogenes*. In Senegal, Diop et al. (2007) isolated 220 LAB strains from traditional fermented foods. Twelve of them were bacteriocin-producing isolates of which two isolates were identified as *L. lactis* subsp *lactis* and *Enterococcus faecium*. In Nigeria, Adetunji and Adegoke (2007) identified *L. plantarum* and *Lactobacillus brevis* as the most dominant bacteriocin-producing LAB strains isolated from West African soft cheese however *Lactococcus lactis*, *Streptococcus lactis* and *Lactobacillus fermentum* were less dominant. In France, Ennahar et al. (1996) identified six bacteriocin-producing LAB isolates from cheese

including three *Lactococcus* strains, two *Enterococcus* strains and one *Lactobacillus* strain which was active against *Listeria* organism. In Belgium, Enan et al. (1996) isolated one strain of *L. plantarum*, from dry sausage, exhibiting an inhibitory activity against *L. monocytogenes*, *B. cereus*, *Clostridium perfringens* and *Clostridium sporogenes*. In Brazil, Moreno et al. (2000) isolated 15 strains of *L. lactis* (14 *Lactococcus Lactis* subsp. *lactis* and one *L. lactis* subsp *cremoris*) from local dairy products that were able to produce bacteriocins inhibited *Listeria monocytogenes* and *S. aureus*. In Turkey, Erdogru and Erbilir (2006) isolated two strains from foods

Table 3. Sources and numbers of the identified bacteriocin-producing lactic acid bacterial strains.

Total No.	Origin	Species
13	Kareish cheese ⁽¹⁾ , Sheep milk ⁽¹⁾ , buffalo's milk ⁽³⁾ , goat milk ⁽¹⁾ , Domiati cheese ⁽³⁾ , Rayeb milk ⁽¹⁾ , sausage ⁽¹⁾ , silage ⁽²⁾	<i>Lactobacillus jensenii</i>
12	Sheep milk ⁽¹⁾ , goat milk ⁽¹⁾ , buffalo's milk ⁽¹⁾ , yoghurt ⁽³⁾ , Ras cheese ⁽³⁾ , sausage ⁽¹⁾ , Rayeb milk ⁽²⁾ .	<i>Lactobacillus acidophilus</i>
7	Sheep milk ⁽¹⁾ , goat milk ⁽¹⁾ , Domiati cheese ⁽¹⁾ , yoghurt ⁽²⁾ , Ras cheese ⁽¹⁾ , silage ⁽¹⁾	<i>Lactobacillus casei</i> sub sp. <i>Rhamnosus</i>
6	goat milk ⁽¹⁾ , yoghurt ⁽²⁾ , Rayeb milk ⁽¹⁾ , sausage ⁽²⁾ .	<i>Lactobacillus plantarum</i>
6	Domiati cheese ⁽¹⁾ , Rayeb milk ⁽¹⁾ , Ras cheese ⁽⁴⁾ .	<i>Lactobacillus helveticus</i>
6	Kareish cheese ⁽¹⁾ , Rayeb milk ⁽¹⁾ , yoghurt ⁽¹⁾ , sausage ⁽¹⁾ , silage ⁽²⁾ .	<i>Lactobacillus gasserii</i>
5	yoghurt ⁽¹⁾ , goat milk ⁽¹⁾ , buffalo's milk ⁽¹⁾ , Kareish cheese ⁽¹⁾ , Rayeb milk ⁽¹⁾	<i>Lactobacillus reuteri</i>
3	Sheep milk ⁽¹⁾ , Domiati cheese ⁽²⁾	<i>Lactobacillus delbrueckii</i> sub sp. <i>Bulgaricus</i>
1	Kareish cheese ⁽¹⁾	<i>Lactobacillus casei</i> sub sp. <i>casei</i> .
16	Kareish cheese ⁽²⁾ , Sheep milk ⁽¹⁾ , buffalo's milk ⁽²⁾ , Domiati cheese ⁽¹⁾ , Rayeb milk ⁽²⁾ , silage ⁽²⁾ , Ras cheese ⁽⁶⁾ .	<i>Streptococcus thermophilus</i>
3	Sausage ⁽²⁾ , Domiati cheese ⁽¹⁾ .	<i>Lactococcus lactis</i> sub sp. <i>Lactis</i>

(): No. of the identified strains.

identified as *Lactobacillus bulgaricus* and *L. casei* that showed various degrees of inhibitory activity against both Gram-positive and Gram-negative bacteria including *E. coli*, *S. aureus*, *P. aeruginosa*, *Bacillus subtilis*, *Klebsiella pneumonia*, *S. typhimurium* and *Enterobacter cloacae*.

Conclusion

The results of this study indicated that some of environmental LAB are capable of synthesizing inhibitive substance(s) against some pathogenic/spoilage bacteria. These inhibitive substances are proteinaceous in nature and act differently against the tested indicators bacteria. The numbers of Gram-negative bacteria, sensitive to these substances, were more than Gram-positive; however the Gram-positive bacteria were more susceptible to the same bacteriocin if compared to the Gram-negative. These strains would contribute to safety and organoleptic properties of Egyptian local food.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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Full Length Research Paper

Antibiotic potentials and isolation of metabolomes from microorganisms of mesophilic soil of Rajasthan, India

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The present of research work was focused on mesophilic soil samples from four different sites of Jaipur (The Pink City) in Rajasthan. Rajasthan is a semi arid region and famous for its sandy soil, heat and temperature which reach up to 50°C in summers. Twenty nine colonies were isolated from the soil samples on nutrient agar media and three distinct isolates were selected and characterized after analyzing coloration of colony formation and zone of inhibition around and appearance of the colony. On the basis of biochemical tests, the isolates are suspected to belong to the genera, *Bacillus*, *Actinomycetes* and *Staphylococcus*. Antibiotic activity of the isolates was studied against some common nosocomial infection causing pathogens and promising results were observed. Out of 29 colonies, only 3 colonies viz. S-IA, S-IIA and S-IIIC showed appreciable biological activity against the above mentioned 2 test pathogens *E. coli* and *Pseudomonas aeruginosa*. Further, inhibition zones were calculated in S-IA (IZ = 12 mm; AI = 1.5); S-II A (IZ = 42 mm; AI = 4.2) and S-III C (IZ = 12 mm; AI = 1.1).

Key words: Antimicrobial efficacy, nosocomial infections, mesophilic soil, metabolomes, Rajasthan.

INTRODUCTION

In nature, microorganisms are the most widely distributed group of organisms, and abundantly present in soil. A number of continuous methods are in process for search of new bioactive compounds with high commercial value. Soil microbes provide near about 2/3rd of naturally occurring antibiotics, and many of them have great medical significance. A study shows that approximately 82% of the worlds' antibiotics are known to come from

soil origin (Bobbarala, 2012). Antibiotic resistance in pathogens is the major threat for the medical world these days. These antibiotic resistant microorganisms are known as multidrug resistant strains, that is, multidrug-resistant strains (MDRS). Comparative studies show that emergence of MDRs is faster than the search of new drug and antibiotics. Continuous, improper and over usage of antibiotics/drugs is the main reason behind the

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evolution of MDRs. The pathogens get adapted, change their metabolism, alter the morphology accordingly, and prepare themselves against antibiotic mode of action. Due to this reason, new and untouched habitats attract scientists as well as pharmaceutical industries for the microbial strains isolation (Raghunath, 2008; Maragakis et al., 2008; Loomba et al., 2010)

It is also noted in the past years that gram negative antibiotic resistant pathogens are spreading in community. These pathogens are threatening patients in hospitals and communities with multi-drug resistance, also incorporate resistance to first, second and third generations of penicillin's and cephalosporin's (Urban et al., 2003)

Antibiotic resistance in pathogens is the major threat for the medical world these days. These antibiotic resistant microorganisms are known as multidrug resistant, that is, MDRs. Comparative studies show that emergence of MDR strains is faster than the search of new drug and antibiotics (Memish et al., 2012). Continuous, improper and over usage of antibiotics/drugs is the main reason behind the evolution of MDRs. The pathogens get adapted, change their metabolism, alter the morphology accordingly, and prepare themselves against antibiotic mode of action. Due to this reason, new and untouched habitats attract scientists as well as research and development departments of pharmaceutical industries for the isolation and screening of microorganisms for novel antibiotic production (Zoran et al., 2010).

Nosocomial infections (NI) are one of the most critical issues faced by clinicians and practitioners in daily health practices, while dealing with the severely ill patients. In India NIs are becoming a burden for the patient's health as well as for the country's economy, along with prolonged hospitalization time, increased morbidity and mortality. There has been prolonged research on infections associated with clinics and hospitals in India and several concerning facts have been revealed (Emori and Gaynes, 1993).

Exploitation of microorganisms for one beneficial product to another is increasing day by day, and there is a need of constant effort in the field of medical research for finding novel significant microbes. It is still necessary to find novel bioactive compounds of microorganism origin against MDR strains causing Nosocomial or any other sort of infection. Improved methods and strategies should be used to study the uncommon and less studied class of microorganisms for production of effective antibiotics, with reproducible strains known to produce bioactive metabolites.

Therefore, exploration of semi arid microflora for isolation of strains with potential antimicrobial metabolites. The aim of present investigations was isolation of strains from different soils samples of Jaipur District against MDR and Nosocomial infections of clinical prevalence.

MATERIALS AND METHODS

Study location and experiment design

This prospective study was undertaken in Department of Microbiology, JECRC University, Jaipur, India some experiments were also performed at S.P. Institute of Biotechnology and Department of Microbiology, SMS Medical College, Jaipur, India during a period of 8 months (August 2013 to April 2014). GC-MS analysis was performed at Advanced Instrumentation Research Facility (AIRF), School of Physical Sciences (SPS), Jawaharlal Nehru University, New Delhi, India.

A total of four samples of Mesophilic strains were collected from soil of Jaipur region and five Nosocomial infection causing pathogens (NICP/ test pathogens) were acquired from SMS Medical College and Hospital, Jaipur, India. Isolates were obtained mesophilic soil and biological activity was tested against NICP. Compounds responsible for antibiotic activity were identified at AIRF and similarity was match with drug library.

Sample collection

Soil samples were collected in sterile air tight plastic bags by sterile shovel from four locations of Jaipur, Rajasthan, that is, slum area of jawahar circle, SMS hospital, Durgapura and Mansarovar. The samples collected were named as S-I, S-II, S-III and S-IV, respectively. Selection of site was also based on various factors, temperature of the particular area, population load around the area, presence of any organic or inorganic additive/contaminant to the soil of the particular region, ease of repetition of sample collection, ease of accessibility of the area. Soil was collected from 4 cm deep each of these locations. It was then brought to the laboratories and stored at 4°C till further processing.

Isolation of microorganisms

Suspension of all the four soil samples were prepared and followed by serial dilution method. Spread plate technique on nutrient agar plates was used for the isolation of microorganisms. Serial tenfold dilution of mesophilic soil samples were spread on sterile nutrient agar and incubated at 36°C for 24 h. Total 29 isolates were obtained out of which only six were morphologically dominant which were selected for further study.

Identification

Selected six isolated of microbial strains were performed for gram staining. Out of the six isolates, four isolates were gram positive, confirmed by gram staining. All the isolates were assigned a specific number according to sampling location, that is, S-I (Jawahar Circle) S-II (SMS) S-III (Durgapura) S-IV (Mansarovar) and according the isolated strain A, B, C, D. All the isolates were identified by some biochemical tests and microscopic morphological studies and assessment by Bergy's manual. Isolates were checked for the presence of enzyme like catalase, urease, oxidase and hydrolase by qualitatively standard methods.

Fermentation

The colonies screened from the dilutions of soil samples were inoculated in Luria broth media. Shaker treatment for 2 h daily at 700 rpm was provided for 30 days at different temperature ranges. Regular testing of metabolites (primary/secondary) was done after

Table 1. Isolation of microorganisms.

Soil sampling location	Soil sample I.D.	Total no. of bacterial isolates	Isolate I.D.
JC slum area	S - I	7	A,B,C,D,E,F,G
SMS hospital	S - II	8	A,B,C,D,E,F,G,H
Durgapura	S - III	7	A,B,C,D,E,F,G
Mansarover	S - IV	7	A,B,C,D,E,F,G

7, 15, 21 and 27 days.

Isolation of mixture containing bioactive compounds

After 27 days of incubation, each culture was centrifuged at 8000 rpm for 10 min and supernatant was collected separately. Metabolites were extracted using three solvents, that is, benzene, ethyl acetate and chloroform. Supernatant was mixed in 2:1 ratio with each of the three solvents, shaken and allowed to mix properly. The mixture was left undisturbed to allow the separation of the solvent having the dissolved metabolites from the culture. The solvent was then decanted from the culture and allowed to vaporize at 40 to 50°C in the oven. The method used for separating bioactive compounds from extracellular secondary metabolites was liquid-liquid extraction. Further, these extractives were named as LLE-I, LLE-II and LLE-III for benzene, ethyl acetate and chloroform respectively.

Antimicrobial analysis

The dried form of compounds collected was again mixed in 2 to 3 ml of respective solvents. Sterilized circular discs were cut and soaked in solvents containing bioactive compounds. Lawn of five common nosocomial infection causing pathogens, that is, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Escherichia coli* was prepared on nutrient agar.

The antimicrobial activity was then tested against these pathogens using disc diffusion method (Saadoun et al., 2008). Plates were incubated at 38°C for 48 h.

Test pathogens

Pure cultures of five test pathogens were obtained from Department of Microbiology, JECRC University, Jaipur and SMS Hospital. These included *Pseudomonas aeruginosa* MTCC 7093, *S. aureus* MTCC 7443, *E. coli* MTCC 40, *K. pneumoniae* MTCC 530, and *B. subtilis* MTCC 121 (Table 1).

Microbial isolates were tested against these five bacteria using disc diffusion, well diffusion and cross streak methods (Magaldi et al., 2004; Valgas et al., 2007; Saadoun et al., 2008; Balouiri et al., 2016).

Separation and characterization of bioactive compounds by thin layer chromatography (TLC) and gas chromatography-mass spectrometry (GC-MS)

Centrifugation

Isolates kept for fermentation were centrifuged at 8000 rpm for 10

min. Supernatants of S-IA, S-IIA and S-III C were performed for liquid extraction with benzene, ethyl acetate and chloroform as LLE-I, LLE-II and LLE-III respectively and processed for antimicrobial screening and three active rich fractions were collected and stored at 4°C for further studies.

Thin layer chromatography (TLC)

Glass plates of 18 cm x18 cm were used to perform TLC, so that approximately 4 samples could be run together. Slurry was made with silica gel and water. Mixing and shaking of silica gel in water should be proper for homogenous and adhesive mixture. Thin layer on glass plates were formed and kept for 3 to 4 h on the plain surface for drying and later in the oven at 70°C. The temperature of the oven was raised to 110°C for 1 h for activation of the plates. Taken the plates out and allowed to cool. Mobile phase (solvent) prepared and poured in TLC glass chamber in which plates spotted with extract were placed. The plates were placed in the chamber till it run or develop upto ¾ of the TLC plate and then again kept in oven for drying. Plates were sprayed with specific reagents and spots were observed. The developed plate was baked at 110°C for 30 min and observed under UV light chamber and the displacement of development (mobile phase) and extract were measured and recorded (Sahin and Ugur, 2003).

GC- MS analysis

Shimadzu model QP-2010 plus, column-Rtx -Ms, 30 m x 0.25 mm i.d. x 0.25 µm film thickness was used for detection. Samples were prepared accordingly for analysis. Extracts were collected in 100 ml beaker and mixed with methanol. Mixture was filtered properly to remove any crystal particle. Homogenous solution was collected in ependrof after testing it with microinjection. Samples were loaded in injector and processed. Chromatograms with compounds detected in solvent were recorded and compared with compound library (Ceylan et al., 2008).

RESULTS

Isolation, selection and biochemical testing were performed for primary screening of microorganisms. Main objective is to find a compound which can be useful in manufacturing drug against pathogens which causing nosocomial infections. After plating methods, microbes were selected on the basis of their growth pattern and morphological features. 29 colonies were targeted as different among all the colonies. These 29 colonies were further sub-cultured to isolate as pure and for further

Table 2. Test pathogens.

Test pathogens	MTCC code	Pathogen I.D.
<i>Pseudomonas aeruginosa</i>	MTCC 7093	P1
<i>Staphylococcus aureus</i>	MTCC 7443	P2
<i>Escherichia coli</i>	MTCC 40	P3
<i>Klebsiella pneumonia</i>	MTCC 530	P4
<i>Bacillus subtilis</i>	MTCC 121	P5

Table 3. Extracts containing bioactive compounds.

Solvent/sample	Benzene	Ethyl acetate	Chloroform
	Extract in mg/100 mL		
S-1 A	0.09	0.08	0.04
S-II A	0.17	0.09	0.05
S-III C	0.19	0.18	0.14

Table 4. TLC R_f value and color of spot.

Isolates	R _f value			Colour of spot		
	Benzene	E.A.	Chloroform	Benzene	E.A.	Chloroform
S-1 A	-	0.3	-	-	Dark brown	-
S-II A	0.4	0.13	-	N.D.	Dark orange	-
S-III C	-	-	0.09	-	-	Grayish

testing. To mark which ones were having biological activity against some pathogens as well as nosocomial infection causing pathogens, 29 isolates were tested through different activity test methods. The five test pathogens used were: P1 - *P. aeruginosa* (MTCC 7093), P2- *S. aureus* (MTCC 7443), P3 - *E. coli* (MTCC 121), P4 - *K. pneumonia*, (MTCC 530) and P5 - *B. subtilis* (MTCC 121). 3 colonies (that is, S-IA, S-II A and S-III C) showed biological activity against two test pathogens, that is, (*Pseudomonas aeruginosa* and *E. coli*) out of selected five test pathogens. Further, inhibition zones were calculated in S-IA (IZ = 12 mm; AI = 1.5); S-II A (IZ = 42 mm; AI = 4.2) and S-III C (IZ = 12 mm; AI = 1.1) as recorded in Table 5. All the selected strains were performed for biochemical test and microscopic morphological studies (Table 6) as per Bergey's manual. Isolates were checked for the presence of enzyme like catalase, urease, oxidase and hydrolase by qualitatively standard methods (Table 2).

For secondary screening, the above mentioned 3 isolates were processed through fermentation process with regular shaker treatment for a specific duration and different incubation period and temperature.

Centrifugation followed by extraction with solvents benzene, ethyl acetate and chloroform as LLE-I, LLE-II and LLE-III, respectively (Table 3).

Above strategies and tests confirmed that the isolates S-IA, S-IIA and S-IIIC are having the compounds which need to be targeted. For detailed information the compounds were processed through thin layer chromatography (TLC) (Table 4), contact microbial autography and GC-MS (Figures 2 to 4).

GC - MS study elaborated every bit of information about the compounds extracted from isolates, like molecular weight, RT time, boiling point, structural formula, linear formula, IUPAC name. The results are given in Tables 7, 8 and 9 for S-I A, S-II A and S-III C, respectively. Applications and medical significance of every compound was analyzed with chem library (Ceylan et al., 2008).

GC -MS analysis of S-IA, S-II A and S-III C revealed that these samples possessed seven, seven and two antibacterial compounds, respectively (Table 5). Chromatograms showed the number of detected peaks in each solvent. The RT of the Peaks reveals the presence of different compounds. The area covered by the peaks is



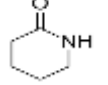
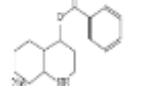
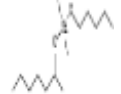


Table 5. Antimicrobial analysis.

Identification of Isolates shown antibiotic activity		Zone of inhibition (cm)				
		Test pathogens				
		P1	P2	P3	P4	P5
Soil samples	S - I A	12	0	0	0	0
	S - II A	0	0	42	0	0
	S - III C	0	12	0	0	0

Table 6. Morphological features and Gram staining.

Sample location	Shape	Colour	Opacity	Elevation	Surface	Consistency/texture	Gram + ve/-ve
S - IA	Filamentous	White	Opaque	Negligible	Rough	Viscid	Positive
S - IIA	Rods	Buff/yellow	Opaque	curve	Smooth	Butyrous	Positive
S - IIIC	Rods	Buff	Opaque	curve	Smooth	Butyrous	Negative

Table 7. Name, retention time (RT), molecular formula, molecular weight, peak area %, structure, nature and biological activity of the compounds present in sample S - I A.

RT	Name of the compound	Molecular formula	Molecular weight	Peak area%	Structure	Nature of compound	Activity
3.454	1H-pyrrole	C ₄ H ₅ N	79	0.47		Aromatic Compounds	Hypolipidemic, antimicrobial, anti-inflammatory and antitumour activities, inhibit retroviral reverse transcriptases
9.608	Cyclobutene-3,4-dione	C ₄ H ₂ O ₂	94	0.30		Clear colorless liquid	Antibacterial, Antifungal
12.983	2-Piperidinone	C ₅ H ₉ NO	99	25.15		Heterocyclic compounds	Antibacterial, Antifungal
13.977	Perhydroquinolin-4-yl ester	C ₁₆ H ₂₁ NO ₂	259	0.11			Antibacterial
33.775	Silane	C ₁₄ H ₃₂ O ₂ S _i	260	0.25		Colourless, flammable gas	Antibacterial
46.172	Pentacosane	C ₂₅ H ₅₂	352	0.27		Aliphatic Hydrocarbon	Antibacterial
28.357	Palmitic acid	C ₁₅ H ₃₀ O ₂	242	0.57		Fatty acid (saturated)	Inhibits phagocytosis Antibacterial

directly proportional to the amount of compound present in the solvent. The peaks were selected and compared with standard which automatically generated the list of compounds. From the consortium of compounds detected and we focused on the ones which could be responsible for antibacterial, antimicrobial, and antifungal activities. S-IA showed seven prominent compounds with

potentials antibacterial activity (Table 7). 1H-pyrrole show anti-inflammatory and antitumor activity, and inhibits retroviral reverse transcriptase activity. Similarly, cyclobutene-3,4-dione, 2-piperidinone, perhydroquinolin-4-yl ester, silane, pentacosane, palmitic acid compounds show antifungal activities. Heneicosane, heptadecane, nonacosane, pentadecane, pentacosane, tricosane and

Table 8. Name, retention time (RT), molecular formula, molecular weight, peak area %, structure, nature and biological activity of the compounds present in sample S - II A.

RT	Name of the compound	Molecular formula	Molecular weight	Peak area%	Structure	Nature of compound	Activity
26.250	Heneicosane	C ₂₁ H ₄₄	296	21.34		Aliphatic hydrocarbon	antibacterial
16.730	Heptadecane	C ₁₈ H ₃₈	254	5.97		Aliphatic hydrocarbon	antibacterial
23.711	Nonacosane	C ₂₉ H ₆₀	408	1.12		Aliphatic hydrocarbon	antibacterial
27.023	Pentadecane	C ₁₆ H ₃₄	226	0.92		Aliphatic hydrocarbon	antibacterial
28.55	Pentacosane	C ₂₅ H ₅₂	352	0.72		Aliphatic hydrocarbon	antibacterial
17.717	Tricosane	C ₂₃ H ₄₈	324	17.708		Aliphatic hydrocarbon	antibacterial
18.875	Hexadecane	C ₁₆ H ₃₄	226	3.5		Aliphatic hydrocarbon	Antibacterial, antioxidant

Table 9. Name, retention time (RT), molecular formula, molecular weight, peak area %, structure, nature and biological activity of the compounds present in sample S - III C.

RT	Name of the compound	Molecular formula	Molecular weight	Peak area%	Structure	Nature of compound	Activity
22.269	Dibutyl phthalate	C ₁₆ H ₂₂ O ₄	278	6.95		Colorless to faint yellow oily liquid	Antimicrobial larvicidal
24.474	Heneicosane	C ₂₁ H ₄₄	296	5.49		Aromatic compound	Antimicrobial
29.498	1-Heptacosanol	C ₂₇ H ₅₆ O	396	19.12		fatty alcohols	Antibacterial Antiseptic Chemotherapeutic

hexadecane were detected in sample S-IIA (Table 8). There were two common attributes: all the compounds are aliphatic hydrocarbons, and all possess antibacterial activities. The later makes them responsible for acting against nosocomial infection causing pathogens. The samples contained variable amounts of extracts. Heneicosane, tricosane and heptadecane are the major constituents among all biological active compounds found in the extracts.

Three of the compounds that showed antimicrobial and anti-bacterial activities, which were found in S - III C sample (Table 9), also have larvicidal, antiseptic and chemo-therapeutic activities. All these facts make the isolate to fight Nosocomial infection causing pathogens. Dibutyl phthalate is colorless to faint yellow oily liquid, Heneicosane is aromatic compound and 1-Heptacosanol is of fatty acid nature. From the experimental results we can concluded that the detected compounds in active rich fractions of S-IA, S-II and S-III C were responsible for bioactivity against infection caused by nosocomial pathogens.

DISCUSSION

Total 29 microbial strains were isolated from different

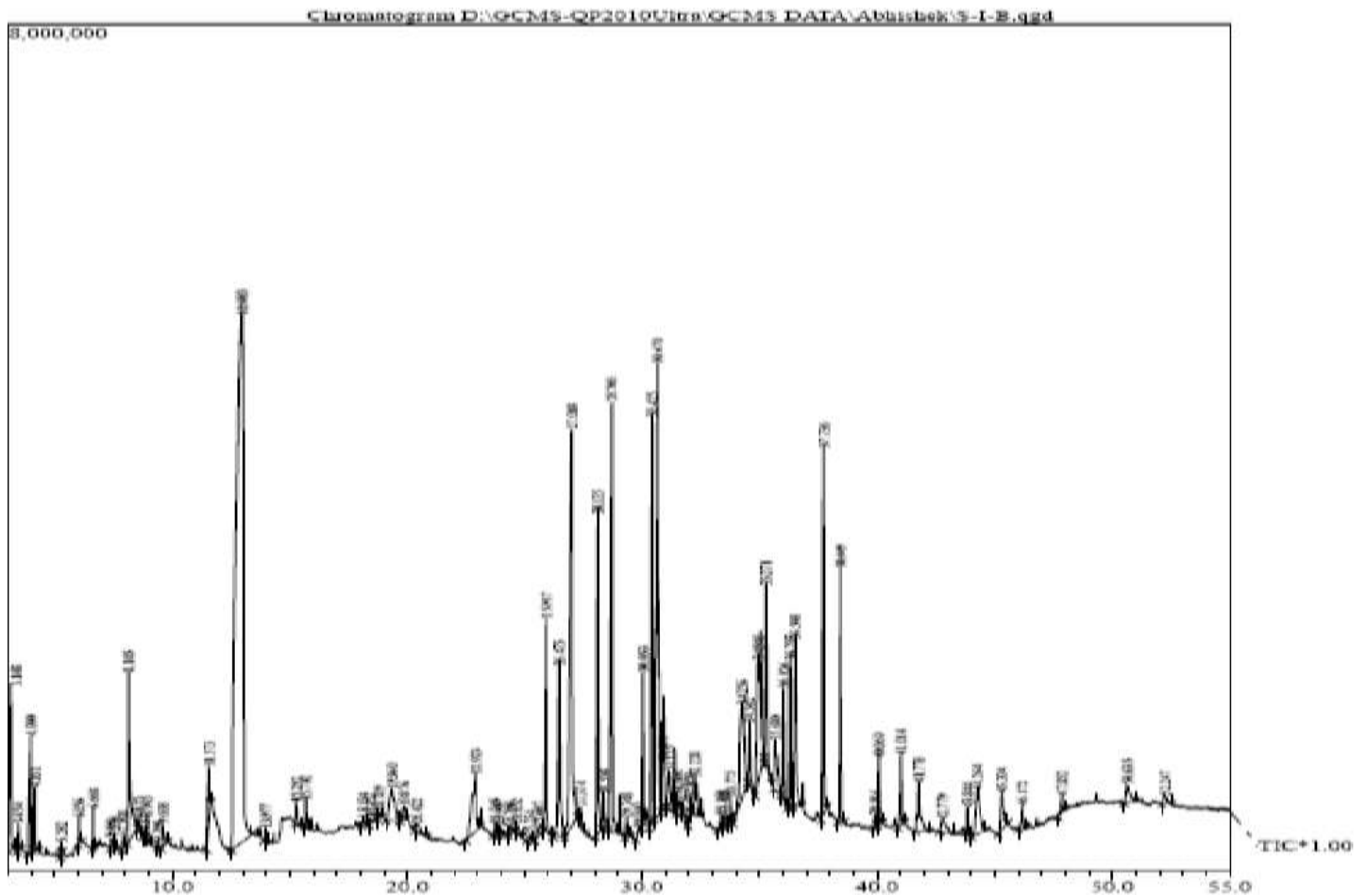
locations of Rajasthan for screening of antimicrobial efficacy against Nosocomial infections causing pathogens. These isolates were performed for antimicrobial screening. Out of which, three were selected for present studies possessing antimicrobial potentials. Further, these selected strains were examined for morphological and biochemical test using Bergeys manual which indicates that these are gram positive bacteria and belong to genus *Bacillus* (Shirling and Gottlieb, 1966). All the three isolates showed appreciable efficacy. S-IA, S-IIA and S-IIIC showed biological activity against selected test pathogens *E. coli* and *P. aeruginosa*. Further, inhibition zones were calculated in S-IA (IZ = 11 mm; AI = 1.5); S-II A (IZ = 42 mm; AI = 4.2) and S-III C (IZ = 12 mm; AI = 1.2) (Figure 1).

Further, studies reveals that active rich fractions showed antimicrobial efficacy against gram negative bacteria and it is noteworthy to mention that the activity index is more than one. The inhibition zone of one of them is higher than 40 mm against *P. aeruginosa*. Pseudomonadaceae has received huge attention in last year's due to its role as pathogenic microorganisms in an increasing number of clinical syndromes (Robin and Janda, 1996).

This pathogen cause various infections and diseases in



Figure 1. Clear zones showing antimicrobial activity against lawn of test pathogens.



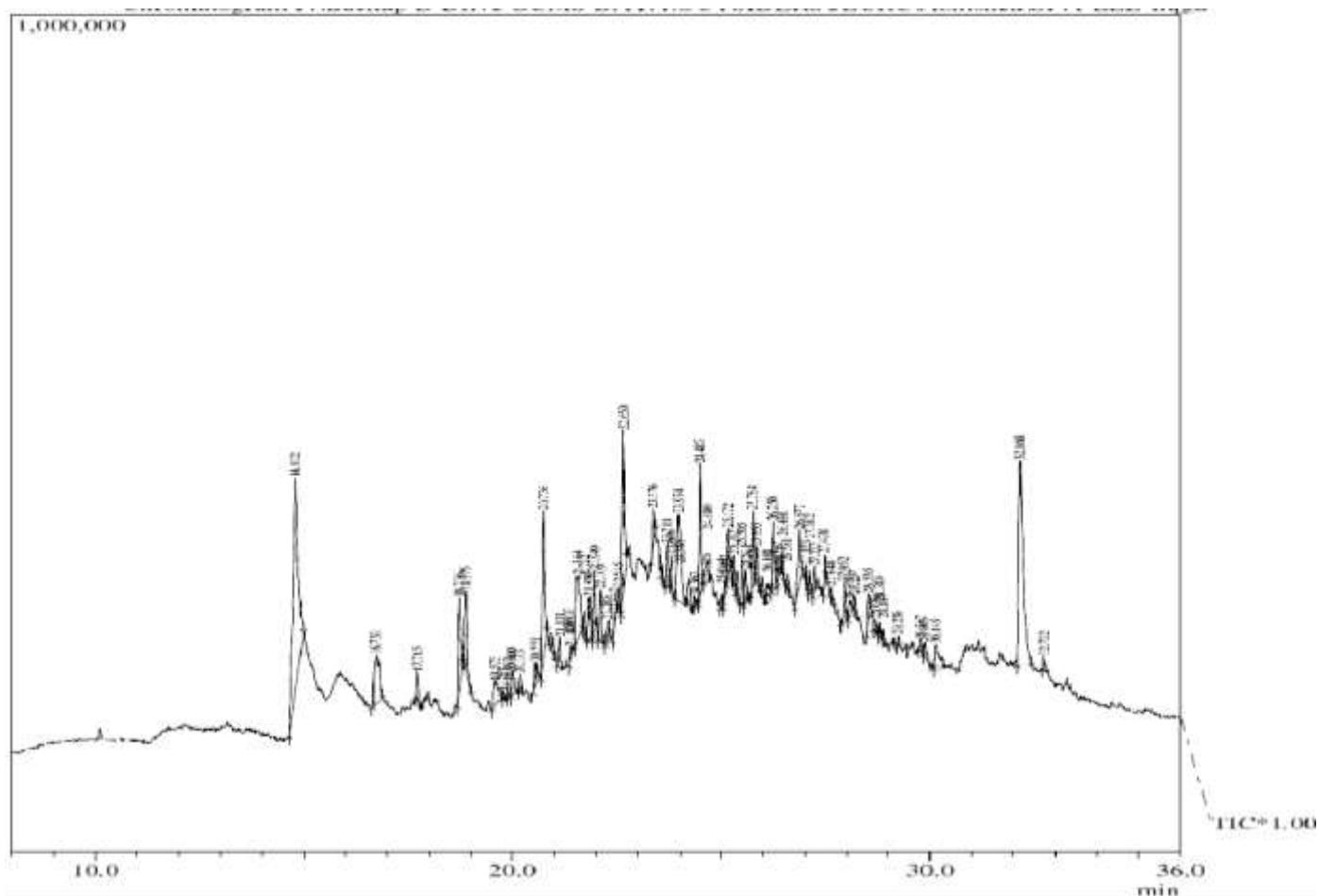


Figure 3. Chromatogram showing peaks of extract S - II A detected in GC-MS analysis.

also been noted as an important nosocomial pathogen (Denton and Kerr, 1998). The treatment of infections caused by this microorganism is difficult because *P. aeruginosa* is frequently resistant to almost all the widely used antibiotics (Vartivarian et al., 1994; Liu et al., 1995; Skaehill, 2000; Krueger et al., 2001). Therefore, production of antibiotics from isolated strains can be further used for large scale production of antimicrobial compounds especially against Nosocomial infections causing pathogens.

In this work, we have shown that a total extraction of secondary metabolites from culture by using different solvents revealed the diversity of compounds produced by the soil isolates. The extraction with different solvents was used to test the biological activity and to predict which class of compound was secreted in culture and could be responsible for the activity. Further, the extracts were processed for GC - MS analysis for characterization of compounds present, along with retention time, melting point, boiling point, structure, molecular weight, and

molecular formula. GC-MS analysis of S-IA strains showed a total of seven peaks. The compounds were 1H-pyrrole (0.47%), cyclobutene 3,4 dione (0.30%), 2-Piperidinone (25.15%), Perhydroquinolin -4-yl ester (0.11%), Silane (0.25%), Pentacosane (0.27%), Palmitic acid (0.57%). S-II A also showed a total of seven peaks. The compounds are Heneicosane (21.34%), Heptadecane (5.97%), Nonacosane (1.12%), Pentadecane (0.92%), Pentacosane (0.72%), Tricosane (17.70%), Hexadecane (3.5%). S-III C strain showed two peaks.

The compounds are Dibutyl phthalate (6.95%), Heneicosane and 1-Heptacosanol (19.12%). Thus, high burden of multidrug resistant pathogens in the current world there is increasing interest for searching of effective antibiotics from soil microflora from diversified ecological niches (Rajasekar et al., 2012). All the seventeen compounds possess antibacterial, antifungal and antioxidant activity. Antibiotics are important bioactive compounds for the cure of infectious disease. Due to emerging of multiple drug resistant, it is difficult to cure

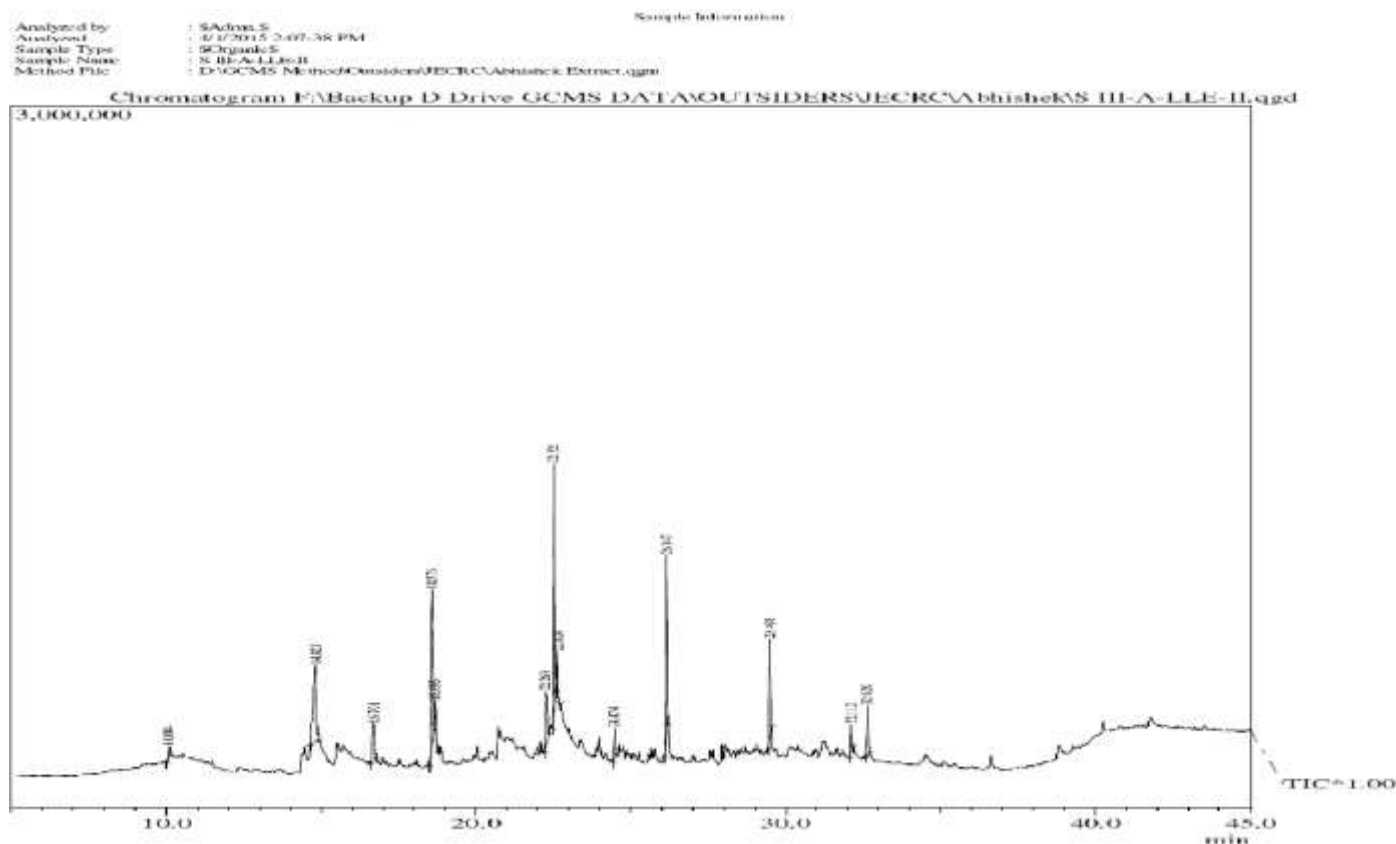


Figure 4. Chromatogram showing peaks of extract S - III C detected in GC-MS analysis.

and get effective treatment for infectious disease.

Conclusion

In the present investigations, attempts were made to isolate 29 strains from soil of Rajasthan and screened for antimicrobial efficacy. Three isolates were found potential antimicrobial efficacy. Further, such isolates were performed for thin layer chromatography for qualitative analysis of secondary metabolites producing micro-organisms. The compounds detected in Sample S-IA, S-IIA and S-IIIC showed biological activity against nosocomial infection caused by pathogens. A total seventeen compounds were identified in the selected strains whereas S-IA strains showed seven compounds, that is, 1H-pyrrole (0.47%), cyclobutene 3,4 dione (0.30%), 2- Piperidinone (25.15%), Perhydroquinolin -4-yl ester (0.11%), Silane (0.25%), Pentacosane (0.27%), Palmitic acid (0.57%), S-II A possessed seven compounds, that is, Heneicosane (21.34%), Heptadecane (5.97%), Nonacosane (1.12%), Pentadecane (0.92%), Pentacosane (0.72%), Tricosane (17.70%), Hexadecane (3.5%) and S-IIIC strain showed three compounds that are Dibutyl phthalate (6.95%), Heneicosane and 1-

Heptacosanol (19.12%). All these compounds possessed therapeutic efficacy against various infections. Isolation of such compounds of microbial origin will provide drugs at lower cost.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Activity of metabolites produced by new strains of *Lactobacillus* in modified de Man, Rogosa and Sharpe (MRS) medium against multidrug-resistant bacteria

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The emergence of nearly untreatable infections caused by multidrug-resistant bacteria has led to a new public health concern in which a need for development of alternative non-antibiotic strategies has become urgent. The activity of metabolites produced by new strains of *Lactobacillus* against multidrug-resistant bacteria was investigated. The objective of this work was to isolate and identify lactobacilli from artisanal kefir by 16S rRNA gene sequencing as well as to evaluate the effect of the growth of *Lactobacillus* species in the Man Rogosa and Sharpe broth, supplemented with Tween 80 at concentrations of 0.6, 0.4, and 0.2 %. Cell Free Supernatants (CFSs) were obtained from these lactobacilli cultures and with them, organic acids (lactic acid and acetic acid) and ethanol were quantified by HPLC followed by the determination of their antimicrobial activities against eight strains of *Staphylococcus aureus*-MRSA and *Klebsiella pneumoniae* KPC strains. The GenBank BLAST analysis revealed that, the isolated lactobacilli belonged to *Lactobacillus paracasei* (n=4) and *Lactobacillus plantarum* (n=5) strains. Increasing concentrations of Tween 80 did not affect the growth of *Lactobacillus* species—significantly when compared to their controls (MRS broth). HPLC analysis of CFSs showed concentrations greater than 18.0, 4.0 and 1.0 g/L for lactic acid, acetic acid and ethanol, respectively. All CFSs were able to inhibit all pathogenic microorganisms evaluated. The percentage of inhibition was on average greater than 88% for MRSA and KPC strains. The antimicrobial activity was dependent on the CFSs tested. Based on these experimental conditions, organic acids and ethanol are likely to be responsible for this antimicrobial activity.

Key words: Antibacterial activity, lactobacilli, cell free supernatants, Tween 80.

INTRODUCTION

The nosocomial infections caused by multidrug-resistant microorganisms (MDR) are among the most serious problems of clinical medicine and pose a major public

health concern (Cecchini et al., 2015). Methicillin resistant *Staphylococcus aureus* (MRSA) and extended spectrum β -lactamase (ESBL) producing *Klebsiella pneumoniae* are

the main bacteria associated with nosocomial infections (Fuji, 2016). MDR develop numerous strategies of resistance to antimicrobial agents and host defenses. Therefore, patients infected by these microorganisms have increased length of hospital stay and higher mortality rate compared to individuals infected with susceptible strains (Spicknall et al., 2013).

According to Cecchini et al. (2015), MDR infections are expected to reach 40 % increase by 2050 that could lead to 10 million deaths per year worldwide. An estimate of between 100 million and 30 billion dollars is being spent annually to treat these kinds of infections, for which therapy is limited to the use of few antimicrobial agents that are often ineffective. In this context, new searches for alternative therapeutic regimen, including the use of probiotics, are needed in order to handle complex infections (Arqués et al., 2015; Alexandre et al., 2014; Alexandre et al., 2013).

Probiotics are defined as living microorganisms, which when administered in adequate amounts, bring a health benefit to the host (Gasbarrini et al., 2016). The probiotics and their byproducts are being used either as prophylactic agents to prevent or delay colonization as well as to combat infections (Şanlıbaba and Güçer, 2015; Prado et al., 2015 Alexandre et al., 2014).

Among the probiotic bacteria, *Lactobacillus* species have received increasing attention because of their specific role in maintaining human health (Gasbarrini et al., 2016). The beneficial action of lactobacilli has been widely described in several reports which focus on their viable cells and cell free supernatants (CFS) (Mariam et al., 2014; Lau and Liang, 2014). The CFS is a well-known source of bioactive compounds such as antioxidants, bacteriocins, surfactants, organic acids, H₂O₂, CO₂, and low molecular weight peptides (Lau and Liang, 2014; Sgibnev and Kremleva, 2016). Their biosynthesis depends on the factors linked to growth such as pH, temperature, O₂ tension, and culture medium composition (Zalán et al., 2010).

Lactobacilli are fastidious microorganisms because they need specific compounds for their growth that are not normally present in other culture media. One of these compounds is Tween 80 that is present Man Rogosa and Sharpe medium (De Man et al., 1960). Tween 80 is a water-soluble ester of oleic acid, which enhances growth and changes the fatty acid composition of cytoplasmic membrane of lactobacilli, making them resistant to environmental stresses (Endo et al., 2006; Li et al., 2011; Broadbent et al., 2014).

In this way, several studies were published relating the presence of Tween 80 in the culture medium to the membrane composition of lactobacilli (Nikkila et al., 1995; Endo et al., 2006; Broadbent et al., 2014). However, the

effect of different concentrations of this surfactant on the metabolism of carbohydrates as well as the antibacterial activity of CFSs against MRSA and KPC strains are still under explored (Mariam et al., 2014; Arqués et al., 2015).

Based on the description above, the aim of this study was (i): To isolate and identify *Lactobacillus* sp. from kefir; (ii) To evaluate the effect of Man Rogosa and Sharpe medium supplemented with 0.6, 0.4 and 0.2 % Tween 80 on growth of *Lactobacillus* sp.; (iii) To obtain cell free supernatants (CFSs) from these cultures; (iv) To quantify lactic acid, acetic acid and ethanol as well as to determine their antibacterial activity against methicillin resistant *Staphylococcus aureus* (MRSA) and *Klebsiella pneumoniae* producing carbapenemase (KPC) strains.

MATERIALS AND METHODS

Isolation of Lactobacilli and phenotypic characterization

Kefir samples of different sources (milk and water) were serially diluted in sterile 0.9 % saline, seeded on Man Rogosa and Sharpe agar, MRS (Lactobacilli MRS Agar, Difco™) and incubated at 30°C for 72 h under 5 % CO₂. Colonies of different morphologies were isolated in MRS plates and characterized by the methodology described in the Bergey's Manual of Determinative Bacteriology (Hammes and Hertel, 2009). Gram-positive, non-sporulating, catalase negative, and rod-shaped bacteria were assumed as being Lactobacilli, and therefore sub-cultured onto MRS agar and then stored into MRS broth with 20 % (v/v) glycerol at -80°C.

Identification of selected Lactobacilli

Lactobacilli isolates (n=9) were identified by Polymerase Chain Reaction (PCR), according to Dos Santos et al., 2015, DNA was extracted using ZR Fungal/bacterial DNA kit (ZymoResearch, Irving, CA, USA). Taxonomical identification was confirmed by sequencing of the PCR- amplified 16S rRNA using the universal pair of primer 8F (5'-CAC GGA TCC AGA CTT TGA TYM TGG CTC AG-3') and the reverse primer 1512R (5'-GTG AAG CTTACG GYTAGC TTG TTA CGA CTT-3'), where Y means C + T and M means A + C. In order, to differentiate *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus*, an average 400 base pair fragments from 16 to 23S intergenic spacing region were also PCR-amplified and sequenced, using the pair of primers Lu-5 (5'-CTA GCGGGT GCG ACT TTG TT-3') and Lac-2 (5'-CCT CTT CGC TCG CCG CTA CT-3').

All PCR reactions were done in a final volume of 25 µL, each containing 0.125 µL of Taq DNA polymerase (New England Biolabs) and 5 pmol of each primer. Sequencing reactions were performed for the forward and reverse strands using the DYEnamic™ET Cycle Sequencing kit (GE Healthcare, Piscataway, NJ, USA) and the DNA sequencing analyzer MegaBACE (GE Healthcare, Little Chalfont, UK). BLAST tool and *Lactobacillus* genome reference sequences (*L. casei* ATCC 334 – AF121200, *L. paracasei* JCM1181- AF182724, *L. plantarum* ATCC 14917 – AF080101, *L. pentosus* ATCC 8041 – U97134, from National Center for Biotechnology Information (NCBI)) were used for identification analysis. Additional sequence alignments were

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performed using APE (A plasmid editor V 2.0). *L. plantarum* ATCC 8014, *L. rhamnosus* ATCC 9595, and *L. casei* ATCC 7469 strains were used as control organisms in the identification process.

Preparation of modified Man Rogosa and Sharpe (MRS) media, standardization of inoculum and growth of *Lactobacillus* sp.

Lactobacillus species were previously cultured onto MRS agar and incubated at 30°C for 48 h. Single colonies were selected and inoculated into MRS broth supplemented with Tween 80 at concentrations of 0.2, 0.4, and 0.6 % (Sigma-Aldrich) (MRS/TW) to obtain turbidities comparable to the 0.5 McFarland standard (10^8 CFU/mL).

Afterwards, these bacterial suspensions were diluted into their respective broths (MRS/TW) to obtain a final inoculum of 10^6 Colony Forming Units per milliliter (CFU/mL). All cultures were incubated at 30°C and samples were analyzed after 6, 9, 12, 24, 48, and 72 h to determine their growth over time. Viable cells of lactobacilli were enumerated and results were expressed as \log_{10} CFU/mL. The MRS broth was used as a control. Parameters related to growth as doubling time (g) and specific growth rate (μ) were calculated (Brizuela et al., 2001; Georgieva et al., 2014).

Organic acids and ethanol analysis by High Performance Liquid Chromatography-HPLC

Lactobacilli cultures in MRS/TW 0.6 % broth were incubated for 48 h and centrifuged at 1,300 g for 15 min. The supernatants were filtered through a 0.22 μ m polytetrafluoroethylene membrane (Chromafil®). The resulting CFS_s were diluted 1:10 in sterile HPLC-grade water followed by the detection and quantification of lactic acid, acetic acid and ethanol.

The analysis was performed on a High Performance Liquid Chromatography column coupled with a refractive-index detector (HPLC-RID) (Agilent, 1200 series), equipped with a binary pump and a diode array detector. Separations were achieved with a column Aminex HPX-87H (300 mm x 7.8 mm, Bio-Rad). Mobile phase was composed of a 5.0 mM sulfuric acid solution at a flow rate of 0.6 mL/min at 35°C. Running time was 25 min and the injection volume of the samples was 20 μ L. The uninoculated MRS broth served as a negative control. The identification of compounds was performed by using DL-lactic acid, acetic acid (Sigma-Aldrich), and ethanol (Merck) standards to compare their retention times with those found in the literature (Sluiter et al., 2008), while the quantification was performed by external calibration with the standards. All samples were analyzed in triplicate.

Determination of the antibacterial activity of cell free supernatants CFS_s

The antibacterial activity of CFS_s was determined against seven *Staphylococcus aureus* methicillin resistant (MRSA) (LFBM 13, LFBM 14, LFBM 17, LFBM 29, LFBM 30, LFBM 34, LFBM 35) and *Klebsiella pneumoniae* producing carbapenemase (LFBM 01, LFBM 02, LFBM 03, LFBM 04, LFBM 05, LFBM 06, LFBM 08) strains. These microorganisms were obtained from stock cultures and maintained in our laboratory (Laboratório de Fisiologia e Bioquímica de Microorganismos (LFBM)).

All microorganisms used in this study showed a resistance phenotype to several antimicrobial agents such as beta-lactams, aminoglycosides, macrolides, fluoroquinolones, tetracycline, chloramphenicol and carbapenems, previously determined by the broth dilution method. *Staphylococcus aureus* ATCC 33591 and *Klebsiella pneumoniae* ATCC BAA 1705 were included in this study as resistant control strains.

These microorganisms were cultured into Mueller Hinton broth. The CFS_s were deposited in sterile 96-wells microplates, and five microliters of test microorganism suspensions were inoculated in each well to give a final concentration of 10^4 CFU/mL. The growth inhibition was demonstrated by optical density at 630 nm using a microplate reader (Thermo plate – TP Reader®) after 24 h incubation at 35°C. Considering the total growth (100 %) in the control wells (MRS broth inoculated), the percentage of growth reduction was attributed to the remaining wells (CFS + bacterium). Negative controls were included after the neutralization of CFS_s with 1N NaOH solution (to pH 7.0). All experiments were performed in triplicate.

Statistical analysis

Data were expressed by mean \pm standard deviation. Analysis was achieved by using the statistical software Graph pad Prism version 5.0. Differences between means were evaluated using one-way and two-way ANOVA. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Lactobacilli identification

The *Lactobacilli* isolates presented unique genetic profiles based on 16S rDNA and 16–23S intergenic spacer region identification. The sequence identities were 99 % similar to *L. paracasei* (GenBank accession N° AF182724) and 99% similar to *L. plantarum* (GenBank accession N° AF080101). These strains were designated as *L. paracasei* (LFBM 01, LFBM 05, LFBM 06, and LFBM 10) and *L. plantarum* (LFBM 02, LFBM 04, LFBM 07, LFBM 08 and LFBM 09).

L. paracasei and *L. plantarum* are known to be associated with a variety of fermented food such as milk and kefir (Prado et al., 2015).

Growth of the *Lactobacillus* species

The growth curves of *Lactobacillus* species exposed to different concentrations of MRS/TW are showed in Figures 1, 2, 3 and 4.

The different concentrations of Tween 80 (0.2, 0.4 and 0.6%) present in the MRS medium did not significantly affect the growth of *L. paracasei* and *L. plantarum* strains, nor did it affect those of *Lactobacillus rhamnosus* ATCC 9595 when compared to their controls or to each other, $p < 0.05$. A discrete growth increase of the *Lactobacillus plantarum* LFBM 02, LFBM 04 and LFBM09 strains in MRS (control) was observed at 24 h. However, this growth was not statistically significant when compared to their respective growth in the MRS/TW media, $p < 0.05$ Figure 2 and 3.

The *Lactobacilli* cultures that remained in the exponential phase for 24 h (0 to 24 h) were used to estimate the linear regression equation. The *Lactobacillus* species showed specific growth rate and doubling time

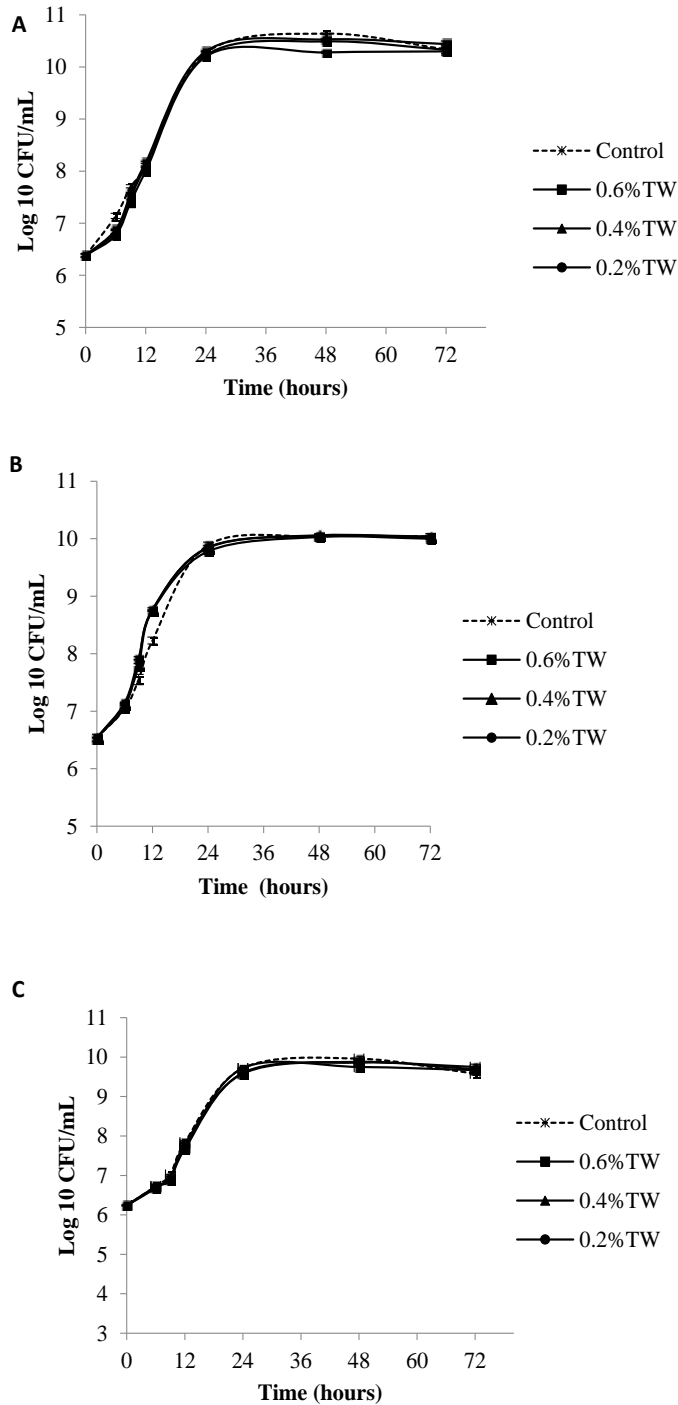


Figure 1. The growth of three strains of *Lactobacillus paracasei* in Man Rogosa and Sharpe-MRS control broths (*) and in the MRS broths supplemented with Tween 80 (TW) at 0.6% (■), 0.4% (▲) and 0.2% (●); (A) LFBM 01, (B) LFBM 05, (C) LFBM 06.

values between 0.12 to 0.21 h⁻¹ and 1.38 to 2.44 h, respectively, Table 1 and 2. When these values were compared to control (MRS), no statistical difference was observed p<0.05. These results are in accordance with some studies that reported a doubling time of about

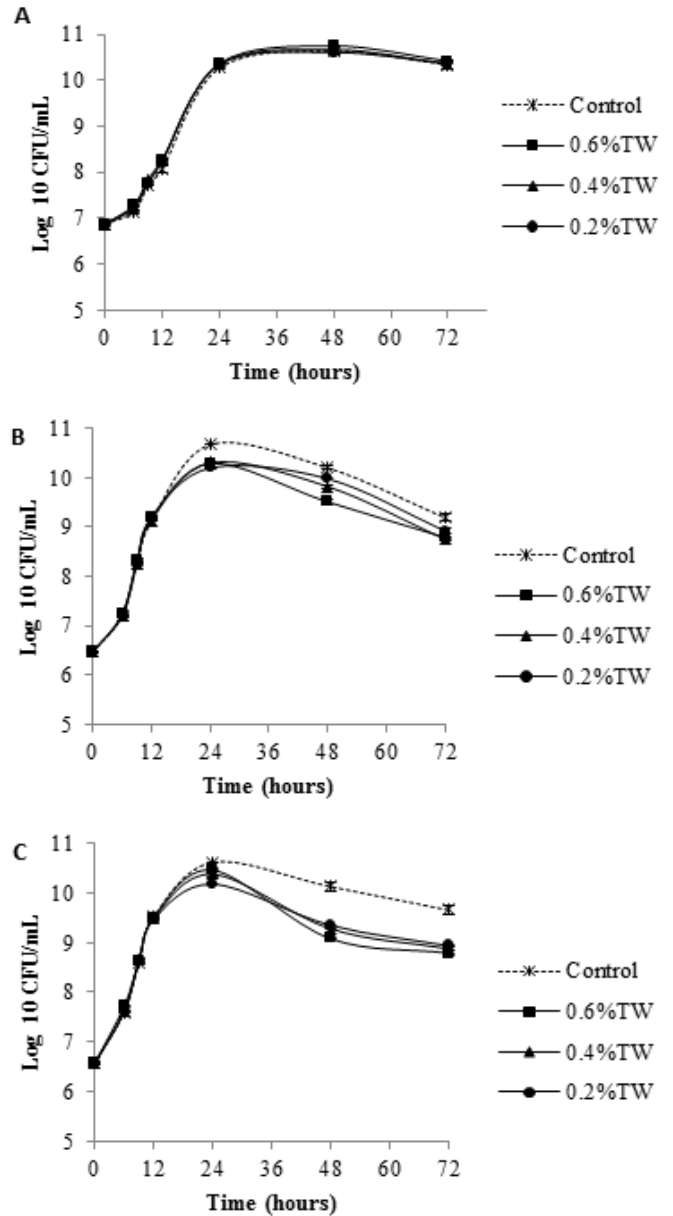


Figure 2. The growth of *Lactobacilli* in Man Rogosa and Sharpe-MRS control broths (*) and in the MRS broths supplemented with Tween 80 (TW) at 0.6% (■), 0.4% (▲) and 0.2% (●); (A) *L. paracasei* LFBM 10, (B) *L. plantarum* LFBM 02, (C) *L. plantarum* LFBM 04.

1 h for lactobacilli (Brizuela et al., 2001; Ayeni et al., 2011; Rezvani et al., 2016).

It is known that, the growth of lactobacilli can be strongly affected by Tween 80 which is often included in the culture media as a growth factor for fastidious bacteria as well as for lactic acid bacteria and *Mycobacterium tuberculosis* (Li et al., 2011; Ghodbane et al., 2014). The oleic acid present in Tween 80 is incorporated into the lipid membranes of bacteria altering the fatty acid composition, fluidity and its permeability

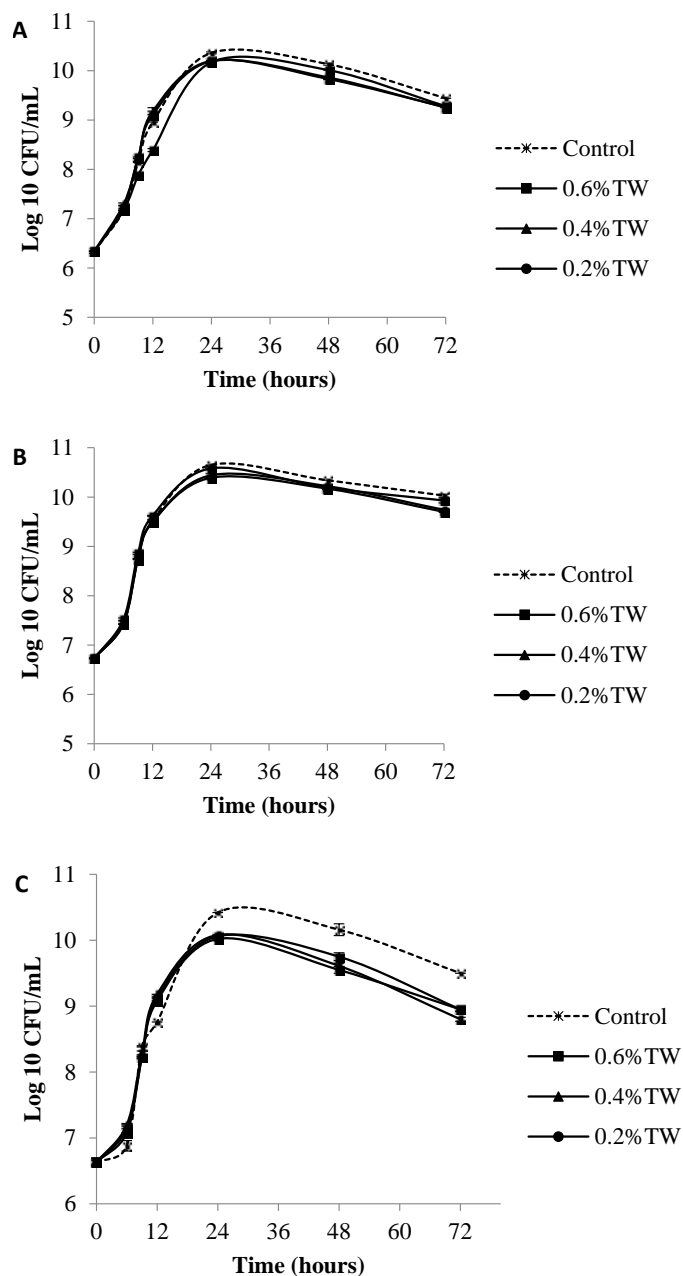


Figure 3. The growth of three strains of *Lactobacillus plantarum* in Man Rogosa and Sharpe- MRS control broths (*) and in the MRS broths supplemented with Tween 80 (TW) at 0.6% (■), 0.4% (▲), and 0.2% (●); (A) LFBM 07, (B) LFBM 08, (C) LFBM 09.

(Nikkila et al., 1995; Kankaanpää et al., 2004). In addition, these fatty acids can still replace the requirement for biotin and be used as a carbon source by some lactobacilli (Li et al., 2011).

In Lactobacilli, the oleic acid is incorporated into membranes through methylation to form dihydrostercularic acid (9,10-methyleneoctadecanoic acid; C19:0cyc9) (Nikkila et al., 1995) which leads to their increasing tolerance to low pH, high bile salts and NaCl

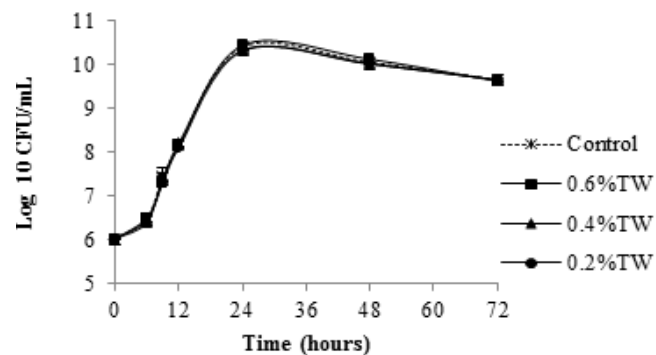


Figure 4. The growth of *Lactobacillus rhamnosus* ATCC 9595 in Man Rogosa and Sharpe-MRS control broths (*) and in the MRS broths supplemented with Tween 80 (TW) at 0.6% (■), 0.4% (▲), and 0.2% (●).

concentrations, as well as their protection against oxidative stress (Li et al., 2011; Hayek and Ibrahim, 2013). The presence of oleic acid in the culture media also stimulates glucosyl transferase secretion as well as accumulation of glycine and betaine, which are amino acids that preserve the structure and function of cellular proteins in environments at high osmolarity level (Guillot et al., 2000; Jacques et al., 1985).

Organics acids and ethanol production

In this work, lactic acid was the major metabolite produced by *Lactobacillus* species whose values ranged from 18.59 to 23.32 g/L, Figure 5. Acetic acid and ethanol were both detected in low quantities Figure 6 and 7. Concentrations greater than 4.0 and 1.0 g/L were produced for acetic acid and ethanol, respectively. We verified that the production of organic acids and ethanol was dependent on the lactobacilli isolated. These results did not present significant differences when compared to each other $p < 0.05$.

Although there is no consensus on the amount of lactic acid that can be produced by lactobacilli, values similar to those found in this study were published by Broadbent et al. (2014) and Ayeni et al. (2011). In addition, the variation of organic acid amounts is dependent on several factors, such as culture conditions, medium composition and in particular, the species of *Lactobacillus* (Srivastava et al., 2015; Zalán et al., 2010). Srivastava et al. (2015) and Coelho et al. (2011) attributed a positive correlation between the presence of Tween 80 in several culture media and the lactic acid production by *Lactobacillus* species. This surfactant promotes the migration of nutrients into the cell by increasing its metabolism, by releasing intracellular enzymes and consequently increasing the production of byproducts (Broadbent et al., 2014).

According to Coelho et al. (2011), higher concentrations

Table 1. Doubling time and specific growth rates of four *Lactobacillus paracasei* strains and *Lactobacillus rhamnosus* ATCC 9595 grown in MRS and MRS supplemented with 0.6%, 0.4 and 0.2% Tween 80.

Strains	Doubling time (g) and growth rates (μ)	Control MRS	MRS supplemented with Tween 80 (TW)		
			TW 0.6%	TW 0.4%	TW 0.2%
<i>L. paracasei</i> LFBM 01	g (h)	1.74 ± 0.04	1.65 ± 0.01	1.67 ± 0.03	1.63 ± 0.04
	μ (h^{-1})	0.17 ± 0.01	0.18 ± 0.01	0.17 ± 0.01	0.18 ± 0.01
<i>L. paracasei</i> LFBM 05	g (h)	1.91 ± 0.03	2.32 ± 0.05	2.22 ± 0.05	2.44 ± 0.02
	μ (h^{-1})	0.15 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.12 ± 0.01
<i>L. paracasei</i> LFBM 06	g (h)	1.68 ± 0.01	1.69 ± 0.02	1.69 ± 0.01	1.65 ± 0.02
	μ (h^{-1})	0.18 ± 0.01	0.17 ± 0.01	0.18 ± 0.01	0.18 ± 0.01
<i>L. paracasei</i> LFBM 10	g (h)	1.77 ± 0.01	1.74 ± 0.04	1.81 ± 0.01	1.76 ± 0.01
	μ (h^{-1})	0.17 ± 0.01	0.17 ± 0.01	0.16 ± 0.01	0.17 ± 0.01
<i>L. rhamnosus</i> ATCC 9595	g (h)	1.38 ± 0.04	1.38 ± 0.03	1.40 ± 0.04	1.39 ± 0.02
	μ (h^{-1})	0.20 ± 0.01	0.21 ± 0.01	0.21 ± 0.01	0.21 ± 0.01

g = doubling time, μ = specific growth rates, TW = Tween 80, LFBM: Laboratório de Fisiologia e Bioquímica de Micro-organismos. Results are expressed by means and standard deviation of two independent trials.

Table 2. Doubling time and specific growth rates of five *Lactobacillus plantarum* grown in MRS and MRS supplemented with 0.6%, 0.4% and 0.2% Tween 80.

Strains	Doubling time (g) and growth rates (μ)	Control MRS	MRS supplemented with Tween 80 (TW)		
			TW 0.6%	TW 0.4%	TW 0.2%
<i>L. plantarum</i> LFBM 02	g (h)	1.73 ± 0.01	1.90 ± 0.00	1.90 ± 0.01	1.95 ± 0.01
	μ (h^{-1})	0.17 ± 0.00	0.16 ± 0.00	0.16 ± 0.00	0.15 ± 0.00
<i>L. plantarum</i> LFBM 04	g (h)	1.87 ± 0.08	1.86 ± 0.00	1.91 ± 0.03	1.95 ± 0.07
	μ (h^{-1})	0.16 ± 0.01	0.16 ± 0.00	0.15 ± 0.00	0.15 ± 0.00
<i>L. plantarum</i> LFBM 07	g (h)	1.80 ± 0.00	1.89 ± 0.01	1.88 ± 0.01	1.89 ± 0.01
	μ (h^{-1})	0.16 ± 0.00	0.16 ± 0.00	0.16 ± 0.00	0.16 ± 0.00
<i>L. plantarum</i> LFBM 08	g (h)	1.50 ± 0.01	1.88 ± 0.00	1.95 ± 0.02	1.98 ± 0.01
	μ (h^{-1})	0.16 ± 0.00	0.16 ± 0.00	0.15 ± 0.00	0.15 ± 0.00
<i>L. plantarum</i> LFBM 09	g (h)	1.95 ± 0.01	2.15 ± 0.01	2.13 ± 0.01	2.17 ± 0.01
	μ (h^{-1})	0.15 ± 0.01	0.14 ± 0.01	0.14 ± 0.00	0.14 ± 0.00

g = doubling time, μ = specific growth rates, TW = Tween 80, LFBM: Laboratório de Fisiologia e Bioquímica de Micro-organismos. Results are expressed by means and standard deviation of two independent trials.

of Tween 80 (higher than 1.4 w/v) were able to decrease the lactic acid production. This observation is likely due to the destruction of the cell membrane and loss of its function, caused by the solubility of lipid bilayer (Qi et al., 2009) as well as by the reduction of lactate dehydrogenase activity (Nagarjun et al., 2005).

In a study conducted by Broadbent et al., 2014, it was observed that the lactic acid production by *Lactobacillus casei* ATCC 334 was influenced mainly by Tween 80 concentration, much more than medium pH or cyclopropane synthase activity, enzyme responsible for the conversion of oleic acid (present in Tween 80) to dihydrosterculic acid. However, when this biosynthesis occurred at a pH of 3.8, the cyclopropane synthase (Cfa) was required in order to protect the cytoplasmic membrane against acid stress. These authors

constructed an ATCC 334 *cfa* knockout mutant and found out that the Cfa inactivation had a negative effect on lactobacilli metabolism, at low pH, mainly on the lactic acid production.

Antimicrobial activity of CFS_s

The results of the antimicrobial activity of CFSs against eight MRSA and KPC strains are presented in Figures 9 and 10. All CFSs from ten lactobacilli cultures showed a stronger inhibitory effect on the growth of test microorganisms. These inhibitory percentages ranged from 65 to 97%. When CFSs were compared to each other no significant difference was observed, except for the CFS from *L. plantarum* 09, which has showed to be

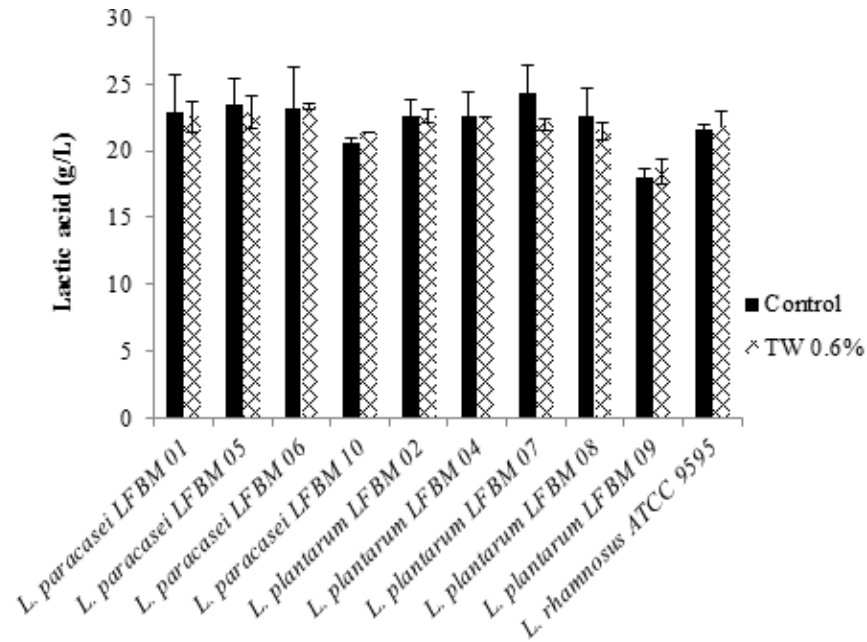


Figure 5. Lactic acid production by ten lactobacilli in Man Rogosa and Sharpe medium-MRS (control) as well as in the MRS supplemented with 0.6% Tween 80 (TW). The analysis was performed with cell free supernatants.

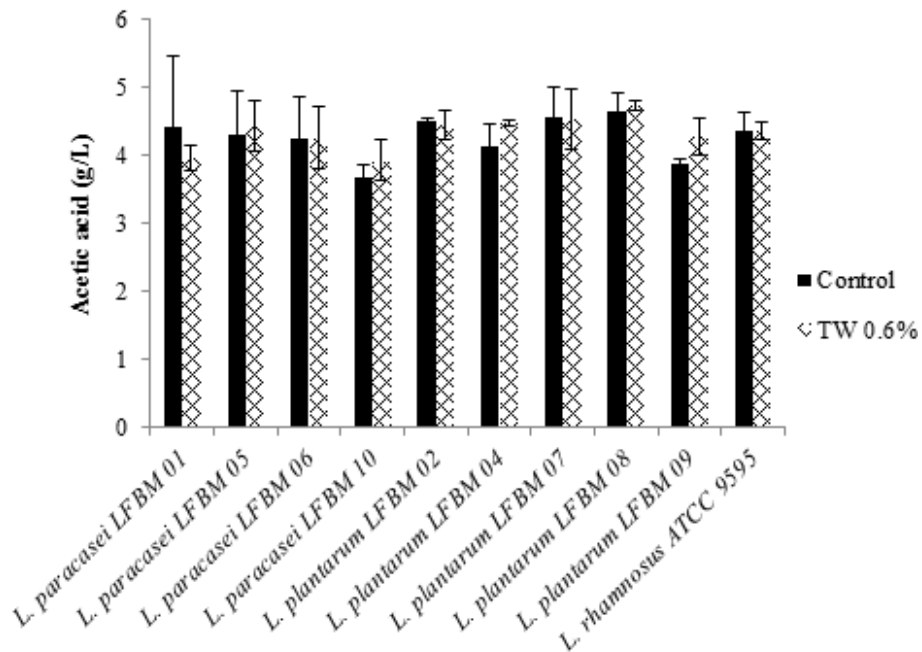


Figure 6. Acetic acid production by ten lactobacilli in Man Rogosa and Sharpe broths (MRS) as well as in the MRS supplemented with 0.6% Tween 80 (TW). The analysis was performed with cell free supernatants.

less active ($p < 0.05$). This antimicrobial activity was dependent on the CFSs tested.

In the present study, a pH decrease was observed after

the incubation into MRS/TW broth for 48 h, Figure 8. This fact suggests that the anti-MRSA and anti-KPC activities of CFSs are closely related to the production of organic

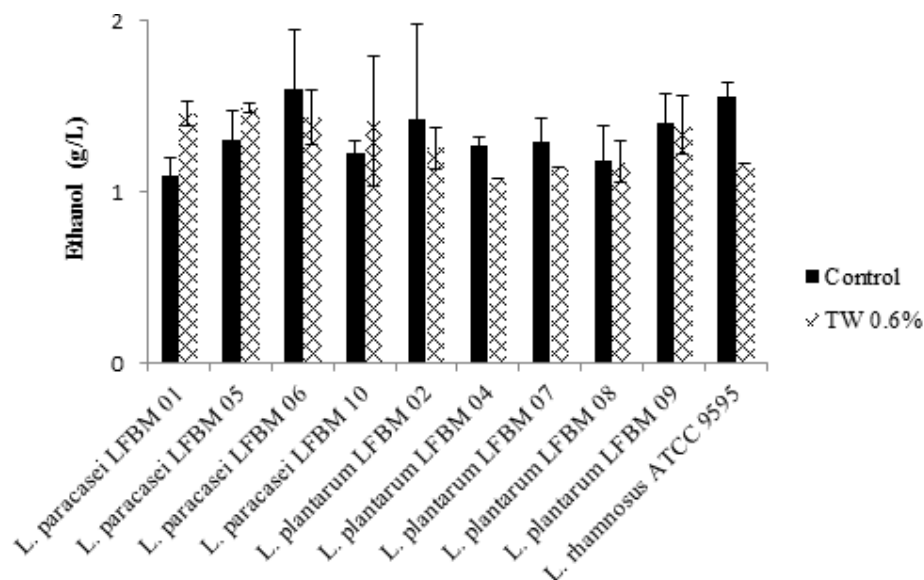


Figure 7. Ethanol production by ten lactobacilli in Man Rogosa and Sharpe broth (MRS) as well as in the MRS supplemented with 0.6% Tween 80 (TW). The analysis was performed with cell free supernatants.

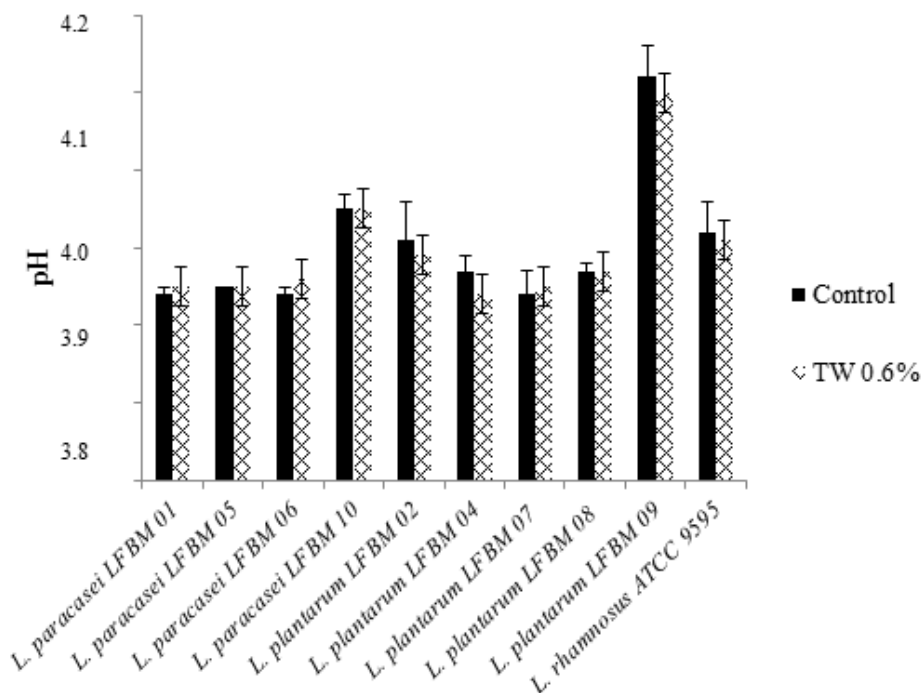


Figure 8. pH values of cell free supernatant of ten lactobacilli cultures in Man Rogosa and Sharpe broth-MRS (control) as well as in the MRS supplemented with 0.6% Tween 80 (TW).

acids, mainly lactic acid. After the neutralization of CFSs, a complete loss of the antibacterial activity was observed. This fact supports that these extracellular antibacterial agents may have an acid nature. The *Lactobacillus* genus

has the ability to produce large amounts of organic acids through the fermentation of carbohydrates besides carbon dioxide, H₂O₂, surfactants and bacteriocins, all with antimicrobial activity (Lau and Liang et al., 2014; Di

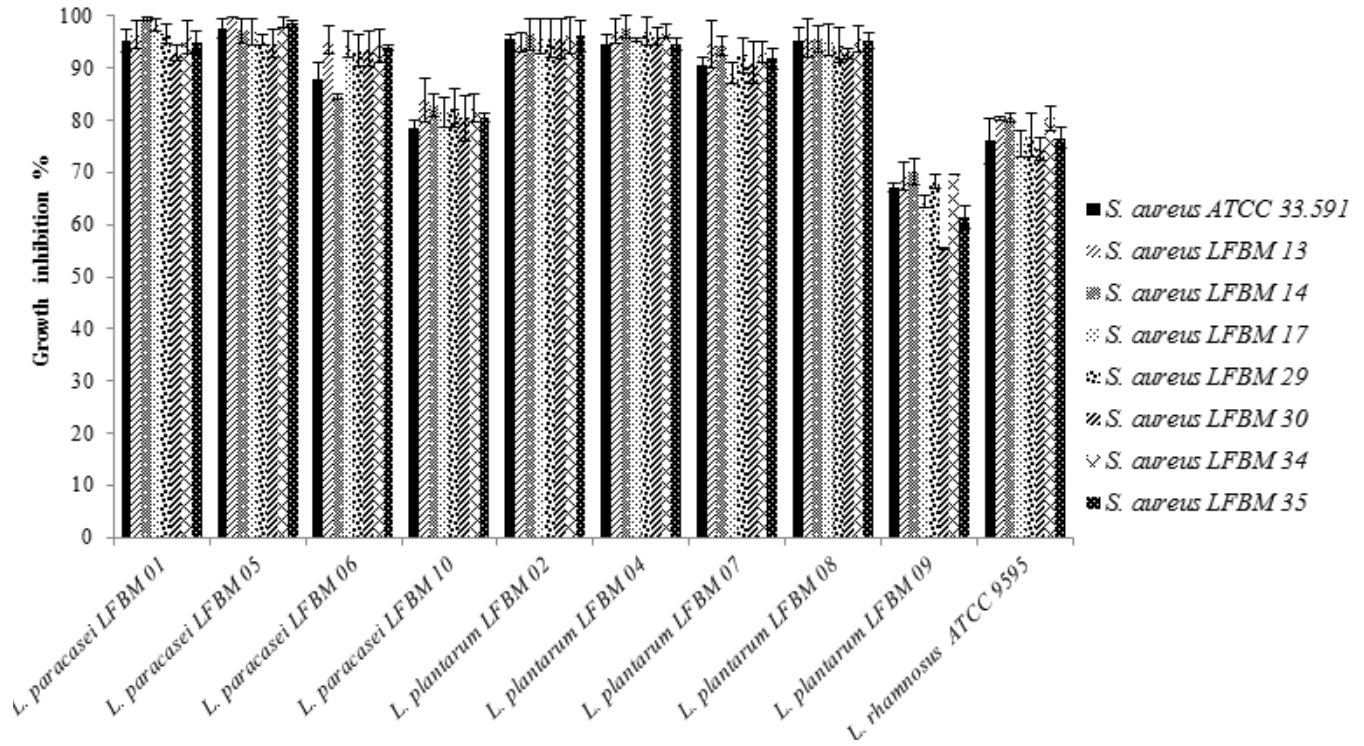


Figure 9. Antibacterial activity of cell free supernatants from ten lactobacilli cultures against eight methicillin resistant *Staphylococcus aureus*-MRSA strains.

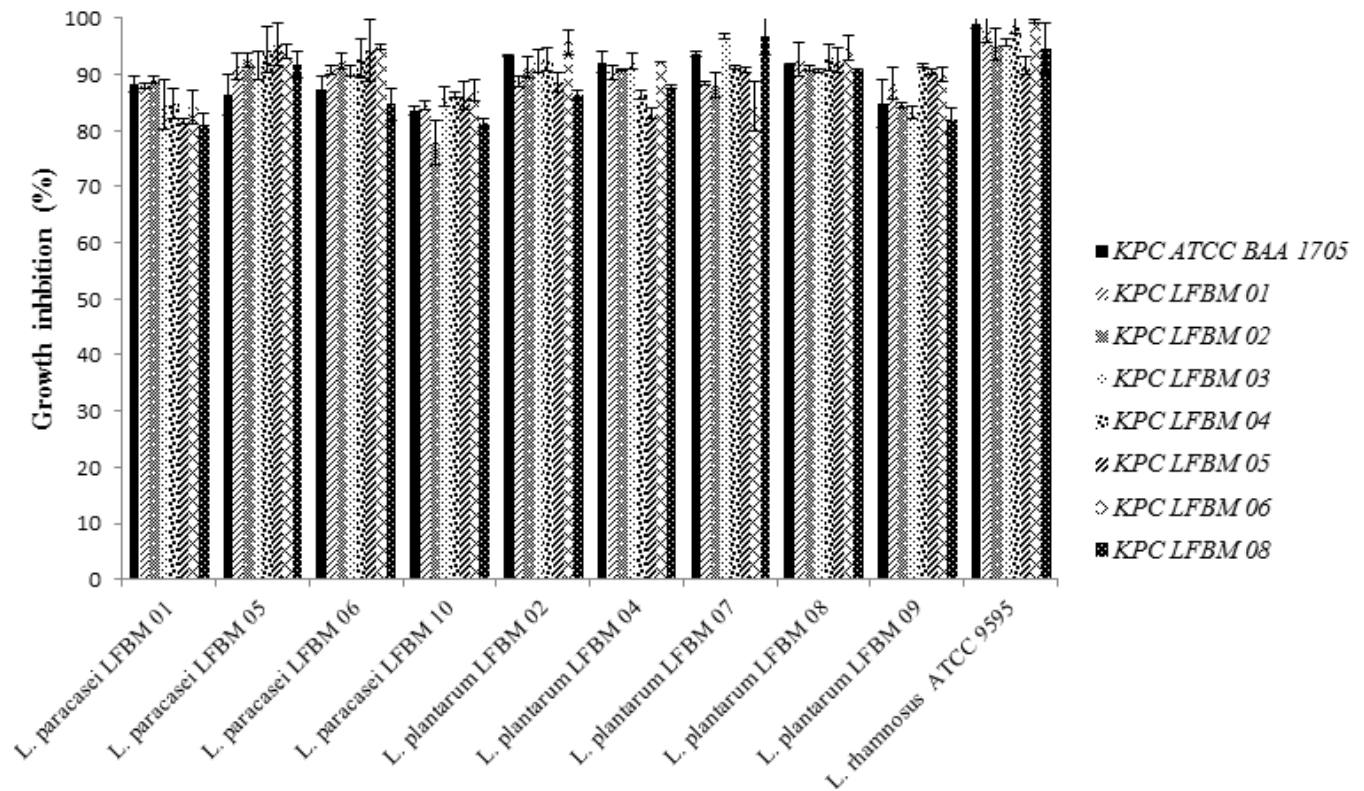


Figure 10. Antibacterial activity of cell free supernatants from ten lactobacilli cultures against eight *Klebsiella pneumoniae* producing KPC carbapenemase strains.

Cerbo et al., 2016; Sgibenev et al., 2016).

The possibility of a bacteriocin being responsible for this activity was ruled out for two reasons: (i) The activity of most bacteriocins drastically decreases during the stationary growth phase (Taheri et al., 2012) (ii) Bacteriocins are unstable in environments below pH 4.0 (Todorov and Dicks, 2005). According to Todorov and Dicks (2005), the supplementation of Tween 80 in the growth medium reduced the level of ST28MS and ST26MS bacteriocins by *L. plantarum*. These authors also observed that these bacteriocins were chemically unstable at low pH levels and that a significant decrease and consecutive loss of their activities occurred after the logarithmic phase. The loss of activity was attributed to different mechanisms such as proteolytic degradation, protein aggregation, adsorption to cell surfaces, and feedback regulation. Moreover, a similar activity pattern was also observed for lactacin B, helveticin J, mesenterocin 5 and enterocin 1146.

The antimicrobial activity of CFSs could be related to the presence of H₂O₂. However, we have used the MRS medium in which lactobacilli do not produce this metabolite. Although the MRS medium is widely used for the growth of lactobacilli, it is less suitable for the studies involving H₂O₂ production, due to the presence of manganese that can catalyze the breakdown of H₂O₂. Thus, some alternative media that lack manganese have been developed, such as MRS without manganese and LAPTg medium. Moreover, the H₂O₂ presence in CFSs would have little or no inhibition in cases when catalase producer organisms, such as *Staphylococcus aureus*, are being evaluated (Pridmore et al., 2008; Martín and Suarez, 2010).

Several studies have been published on antimicrobial activity of lactic acid produced by lactobacilli against pathogen microorganisms (Şanlıbaba and Güçer, 2015; Arqués et al., 2015; Sgibnev and Kremleva, 2016). The antimicrobial activity of organic acids is directly related to the pH reduction and its ability to dissociate. The undissociated forms of these acids are presumed to penetrate the bacterial membrane and dissociate within the cell. As bacteria maintain a neutral cytoplasm pH, the efflux of protons will consume cellular ATP and result in energy depletion (Wang et al., 2015).

The ability to produce large quantities of organic acids, mainly lactic acid through the fermentation of carbohydrates and consequent pH decrease, are fundamental for the antimicrobial activity of *Lactobacillus* species.

Conclusions

According to our results, we conclude that the products from the metabolism of lactobacilli isolate were responsible for the antibacterial activity observed in our study. These important activities against both MRSA and KPC-producing strains may be an important tool towards

new searches for therapy regimen against infections caused by these microorganisms.

It is possible that lactic acid, which was produced in higher amounts, may have played an important role on this activity together with acetic acid and ethanol. Moreover, the presence of other bioactive compounds present in the culture media may have acted synergistically to achieve such activities. Thus, further studies are necessary before new therapies can be implemented.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of nutritional parameters and NaCl concentration on phosphate solubilization potential of *Penicillium purpurogenum* Stoll isolated from paddy field

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Phosphorus (P) is the major essential macronutrient of plants. But its availability in Indian soil is relatively low due to high rate of P fixation. The use of phosphate solubilizing microorganism (PSM) to solubilize the fixed form of P is economically reasonable and ecologically safe as compared to chemical phosphatic fertilizers. Fungi have been reported to possess greater ability to solubilize insoluble phosphate than bacteria. However, phosphate solubilizing efficiency of PSM is found to be affected by different environmental factors. The main objective of this work was to optimize different process parameters for the solubilization of rock phosphate (RP) by the phosphate solubilizing fungus, *Penicillium purpurogenum* Stoll (NFCCI 3788) isolated from paddy field. The concentration of soluble P release by the isolate was tested in Pikovskaya's broth supplemented with RP. Glucose (169 µg/ml) and sucrose (153 µg/ml) significantly promoted P solubilization as compared to other carbon sources. Ammonium sulphate was found to be optimum for maximum RP solubilization. Change in medium pH and mycelial dry weight were also recorded in all the tested groups. The fungus showed different levels of phosphate solubilization under different NaCl concentration tolerating maximum upto 6% sodium chloride concentration.

Key words: *Penicillium purpurogenum* Stoll, carbon source, nitrogen source, optimization, rock phosphate, Chhattisgarh.

INTRODUCTION

Phosphorus (P), the second most essential element after nitrogen, is required by the plants for their growth and development. Apart from its abundance in soil in both organic and inorganic forms, the availability of

soluble P in the soil is limited, explaining the need for the application of chemical phosphatic fertilizers for adequate plant growth. But, use of chemical fertilizers on a regular basis has become costly affair and environmentally

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undesirable. Moreover, large fraction of applied P as chemical fertilizers becomes immobile through precipitation reaction with highly reactive Al^{3+} and Fe^{3+} ions in acidic soil and Ca^{2+} in calcareous or normal soil (Gyaneshwar et al., 2002). Natural phosphate rocks have been recognized as a valuable alternative for synthetic P fertilizers. Conventionally, RP is processed through chemical methods that increases fertilizer cost and makes the environment worse (Panda et al., 2008). The involvement of soil microorganisms in dissolution of fixed forms of P and solubilization of RP is well documented by many researchers (Goldstein, 1986; Kucey et al., 1989; Khan et al., 2007; Xiao et al., 2008). These microorganisms are known as phosphate solubilizing microorganisms (PSM) and include bacteria, fungi and actinomycetes. Several strategies for the solubilization of P are adapted by these organisms, the potential mechanisms either by proton extrusion associated with ammonium assimilation (Roos and Luckener, 1984), or by organic acid production (Cunningham and Kuiack, 1992). The availability of P in soil is regulated by several environmental factors (Hameeda et al., 2008; Srividya et al., 2009), such as nutritional parameters, physiological functions and growth dynamics of the microorganisms (Chen et al., 2006). Therefore, PSMs can be very effective in solubilizing the insoluble phosphate with (Vassilev et al. 1996) or without (Illmer and Schinner 1995) organic acid production. Furthermore, in most studies, ammonium was found to be better nitrogen source than nitrate (Asea et al., 1988) while in some studies, nitrate was reported as better nitrogen source (Relwani et al., 2008). The solubilizing ability has also been influenced by the P source (Nahas, 1996). These findings suggest that phosphate solubilization was affected by various carbon and nitrogen sources. Moreover, the performance of PSM has also been found to be severely affected by stressors (Yadav et al., 2010) such as low and high temperature, pH, salinity, etc. These stressors affect the plant physiology and growth and the activity of plant beneficial microbes including PSM (Mussarat and Khan, 2014). Thus, it seems to be important to evaluate the effect of different factors on solubilization of P by the fungus. The main objective of this work was to examine the effect of carbon and nitrogen sources, RP concentration and salinity on fungal growth and solubilization of RP by *Penicillium purpurogenum* Stoll.

MATERIALS AND METHODS

Isolation of fungi

Fungal strain was isolated by serial dilution agar plating method on potato dextrose agar (PDA from Hi Media, India) plates from rhizosphere soil (pH 5.61) of paddy plant from Raipur, Chhattisgarh, India. The PDA plates were incubated at $28\pm 2^\circ\text{C}$ for five days. Based on distinct morphological characteristics, fungal colony was selected and purified by repeated sub culturing. The pure culture was maintained in PDA slants by sub culturing once in a month.

Screening for phosphate solubilization

Qualitative P solubilization assay

The fungal isolate was subjected to qualitative assay for P solubilization using Pikovskaya's Agar (PKA from Hi Media, India) medium. For this, spore suspension of 5-day old fungal isolate cultivated on PDA was prepared in normal saline. Optical density of the culture was adjusted to 0.3 using spectrophotometer (Elico SL27) at 520 nm wavelength. 10 μl suspension of the culture was inoculated on PKA plates in triplicates and incubated at $28\pm 2^\circ\text{C}$ for 5 days. A clear halozone around the colony indicated P solubilization and expressed as solubilization index (SI) which in turn was calculated using the following formula (Premono et al., 1996):

$$\text{SI} = \frac{\text{Colony diameter} + \text{Halozone diameter}}{\text{Colony diameter}}$$

Quantitative phosphate solubilization assay

The fungal isolate was further subjected to second step of selection based on the quantitative P solubilization in Pikovskaya's broth (Hi Media, India) amended with 0.5% of RP/TCP as insoluble P sources. The initial pH of the broth was adjusted to 7.00 ± 0.03 with 1 N HCl and 1 N NaOH using pH meter (Elico LI610). Flasks containing fungal culture were incubated at $28\pm 2^\circ\text{C}$ upto 5th, 7th and 9th days along with uninoculated control. Cultures were harvested after incubation periods by filtering with Whatman no. 42 filter paper in order to record the change in pH and concentration of released phosphorus in the filtrate. Activated charcoal was added in filtrate in order to avoid color produced by the fungal culture. The final pH of the culture filtrate was measured with a pH meter. Soluble phosphate concentration in filtrate was quantified by the vanado-molybdate method (APHA, 1999). It was expressed as $\mu\text{g/ml}$ phosphorus released in culture medium.

Identification of fugal isolate

The fungus was inoculated aseptically on PDA plates and incubated at $28\pm 2^\circ\text{C}$ for five days and assessed for its morphological characteristics. Further, it was characterized through National Fungal Culture Collection (NFCCI), Agharkar Research Institute, Pune, Maharashtra, India.

Effect of different nutritional parameters and salinity on P solubilization

To evaluate the most significant medium constituents affecting phosphate solubilization, three factors viz. carbon, nitrogen and RP concentration were initially chosen to find out the possible best sources. The optimization was performed using one factor at a time (OFAT) to determine optimum conditions. The fungal isolate was inoculated in 250 ml flasks containing 100 ml of Pikovskaya's broth amended with 0.5 g of RP. To study the effect of carbon sources on the P solubilization, six different carbon sources, that is, glucose, sucrose, fructose, maltose, mannitol, xylose and starch along with control (without carbon source) were used. Each carbon source was added at a concentration of 10.0 g L^{-1} . For selection of most appropriate nitrogen source, six different sources, that is, ammonium sulphate (AS), ammonium chloride (AC), sodium nitrate (SN), potassium nitrate (KN), glycine (GL) and urea (UR) along with control (without nitrogen source) were used. Each nitrogen source was added at a concentration of 0.5 g L^{-1} . The efficacy of RP concentration on P solubilization was studied by varying RP

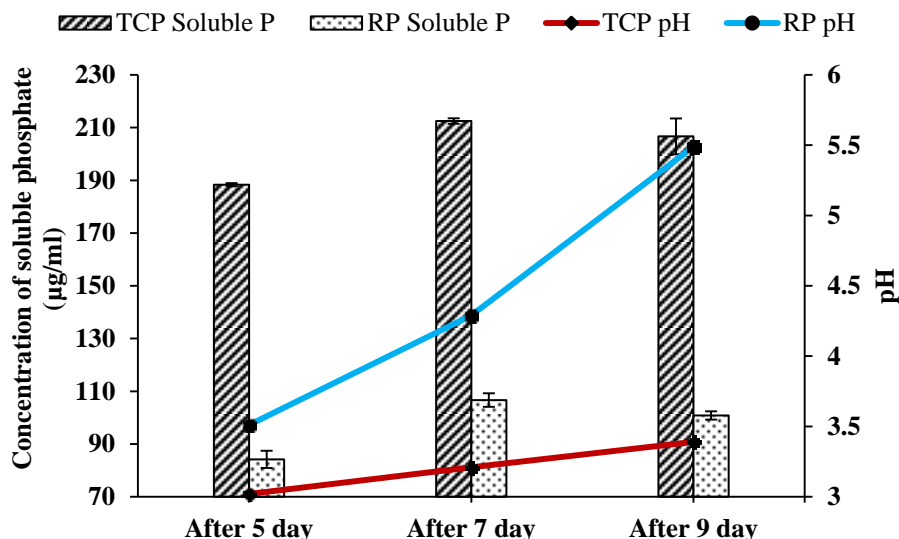


Figure 1. Concentration of soluble P and final pH of the culture medium at different incubation periods (data are means of three replicates \pm SE).

concentrations from 1 to 6%. Flasks containing 0.5% of RP was used as control. The effect of sodium chloride concentration on the P solubilization was performed in seven different concentrations of sodium chloride ranging from 0.2 to 6%. Flasks without sodium chloride were used as control. Initial pH of the medium was adjusted to 7.00 ± 0.03 . Flasks were inoculated with 5% v/v spore suspension (2×10^6 spores/ml) and incubated at $28\pm 2^\circ\text{C}$ for five days. Final pH of the filtrate and soluble phosphate concentration were estimated as mentioned above. The fungal biomass (mycelial dry weight) was determined by weight loss after drying at 105°C in a hot air oven (Tempo SM 1063) for 48 h (Aneja, 2003).

Analysis

Data (three replicates) were subjected to ANOVA and Duncan multiple range test (0.05 level of probability) using SPSS version 16 software.

RESULTS

Fungal strain

The fungus was identified as *Penicillium purpurogenum* Stoll (NFCCI 3788) by NFCCI, Pune, Maharashtra, India.

Solubilization assay

A clear halozone around the colony having SI 2.29 ± 0.03 was observed. Also, a significant quantity of soluble P was detected in medium supplemented with RP/TCP. Phosphate solubilization was accompanied by a decline in pH of the broth. The fungal isolate solubilized 188.33, 212.5 and 206.67 $\mu\text{g/ml}$ of P from TCP with decrease in

pH from 7.0 to 3.02, 3.21 and 3.39 and 84.17, 106.67 and 100.83 $\mu\text{g/ml}$ of P from RP with decrease in pH 3.51, 4.29 and 5.49 after 5, 7 and 9 days of incubation, respectively (Figure 1). However, solubilization was found to be higher in case of TCP as compared to RP.

Effect of carbon source

The results of optimization using different carbon sources are summarized in Figures 2 and 3. The maximum P solubilization was detected with glucose (169 ± 10.83 $\mu\text{g/ml}$) followed by sucrose, maltose, xylose, mannitol and fructose, whereas the minimum level of solubilization was recorded with starch (22.5 ± 1.44 $\mu\text{g/ml}$). Values were significant at the level of $p < 0.001$. A significant ($p < 0.001$) drop in pH from initial value of 7.0 was recorded in all treatments and the value being lowest with mannitol (3.02 ± 0.03). Though high solubilization was observed with glucose, more growth was found with mannitol ($0.19\pm 0.01\text{g}$) and lowest growth was recorded with starch ($0.04\pm 0.003\text{g}$). In the control (without any added carbon source), solubilization of phosphate was not observed while some growth and pH drop was recorded due to the presence of yeast extract in the culture broth.

Effect of nitrogen source

Conversely, a higher concentration of soluble P was found in the case of the control (189 ± 7.26 $\mu\text{g/ml}$). This may be due to the presence of yeast extract in the medium which is a source of nitrogen. In the tested

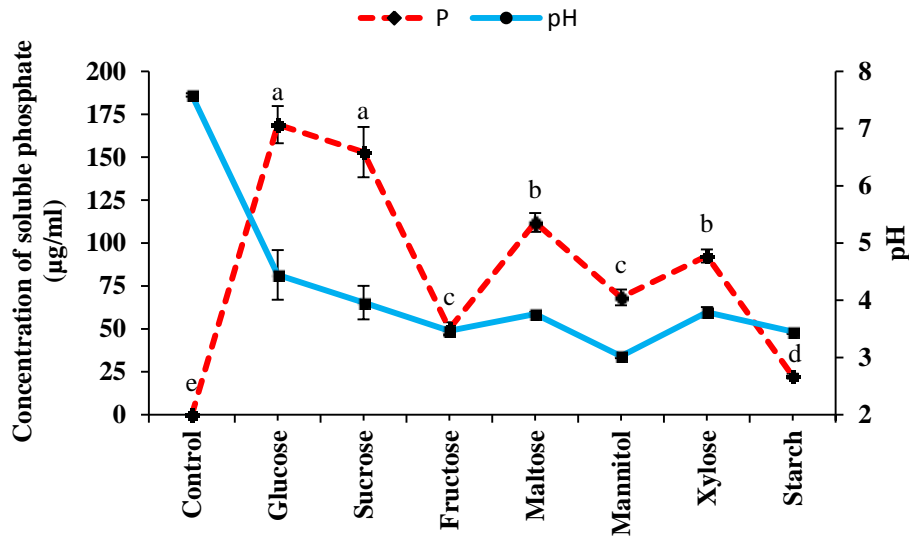


Figure 2. Effect of different carbon sources on concentration of soluble P and pH of the culture medium (data are means of three replicates \pm SE). Means with the same alphabets are not significantly different at $p = 0.05$ (Duncan's).

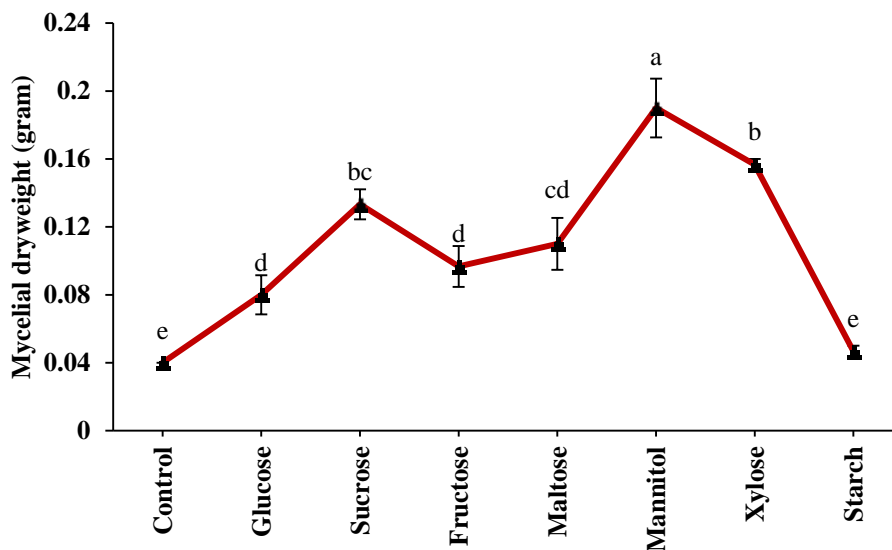


Figure 3. Effect of different carbon sources on mycelial dry weight (data are means of three replicates \pm SE). Means with the same alphabets are not significantly different at $p = 0.05$ (Duncan's).

groups, maximum solubilization was observed in the presence of ammonium sulphate (105 ± 2.88 µg/ml), whereas lowest level of solubilization was observed with sodium nitrate (10.0 ± 1.44 µg/ml). The values were significant at the level of $p < 0.001$. pH of the culture medium significantly ($p < 0.001$) declined from initial pH of 7.0. The lowest value of pH was recorded in the case of the control (3.07 ± 0.03) followed by ammonium chloride (4.23 ± 0.06) (Figure 4). Although, higher soluble P concentration was recorded in the case of the control,

significantly ($p < 0.001$) more growth was observed in the presence of potassium nitrate (0.37 ± 0.003 g) followed by urea (0.35 ± 0.005 g), whereas lowest value was observed for the control (0.13 ± 0.006 g) and sodium nitrate (0.14 ± 0.01 g) (Figure 5).

Effect of RP concentration

A continuous decrease in the level of soluble P was

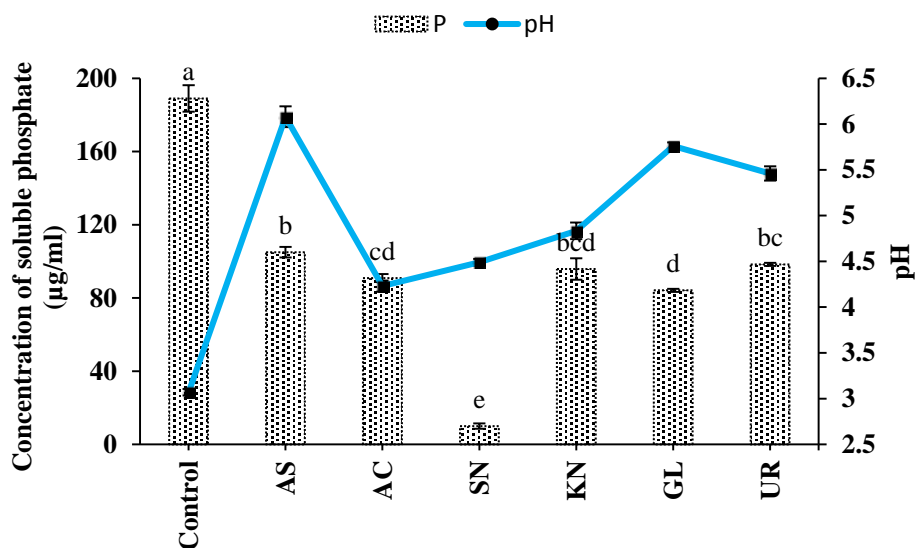


Figure 4. Effect of different nitrogen sources on concentration of soluble P and pH of the culture medium (data are means of three replicates \pm SE). Means with the same alphabets are not significantly different at $p = 0.05$ (Duncan's).

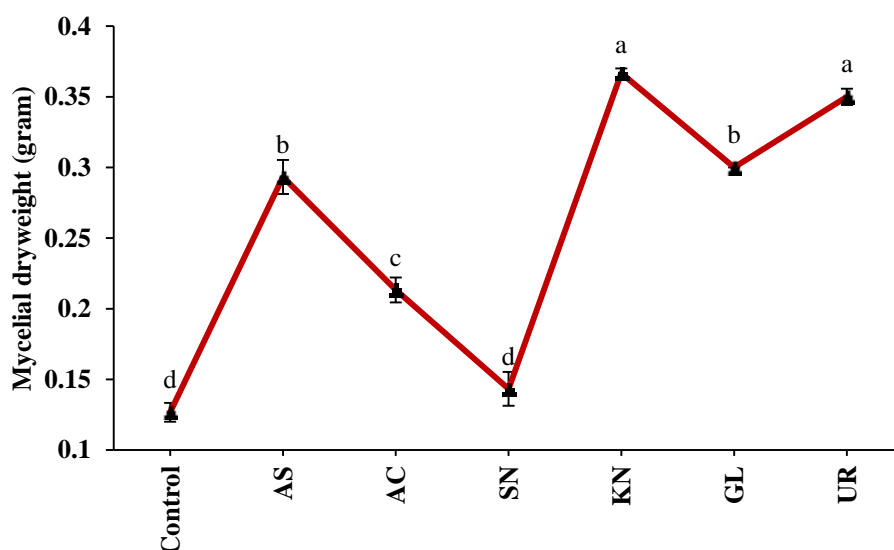


Figure 5. Effect of different nitrogen sources on mycelial dry weight (data are means of three replicates \pm SE). Means with the same alphabets are not significantly different at $p = 0.05$ (Duncan's).

observed with successive increase in RP concentration which is significant ($p < 0.001$) when the amount of RP increased from 1 to 2%. However, the optimum solubilization was observed in the case of control (0.5% RP) and the value was $108 \pm 2.20 \mu\text{g/ml}$. Significant ($p < 0.01$) drop in pH was observed at 5% (4.15 ± 0.13) and 6% (4.17 ± 0.06) (Figure 6). Significant ($p < 0.001$) increase in mycelial dry weight was observed with increasing concentration of RP (Figure 7).

Effect of salinity

The concentration of soluble P was found to be significantly ($p < 0.001$) higher in the case of control ($129 \pm 5.06 \mu\text{g/ml}$) and a subsequent decrease in the concentration of soluble P was observed with the increasing concentration of sodium chloride. Significant ($p < 0.001$) reduction in medium pH of test groups was recorded as compared to the control. The lowest value of

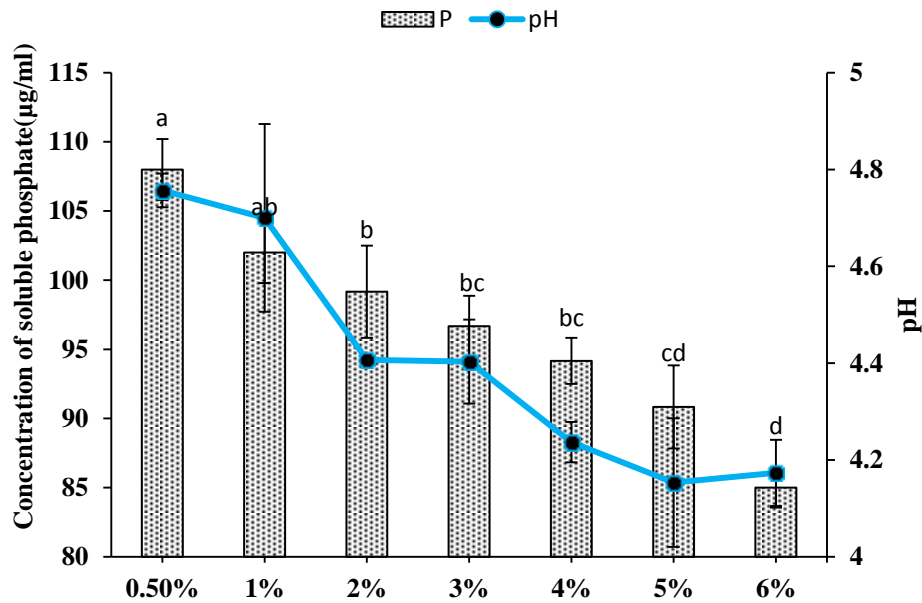


Figure 6. Effect of varying concentration of RP on concentration of soluble P and pH of the culture medium (data are means of three replicates \pm SE). Means with the same alphabets are not significantly different at $p = 0.05$ (Duncan's).

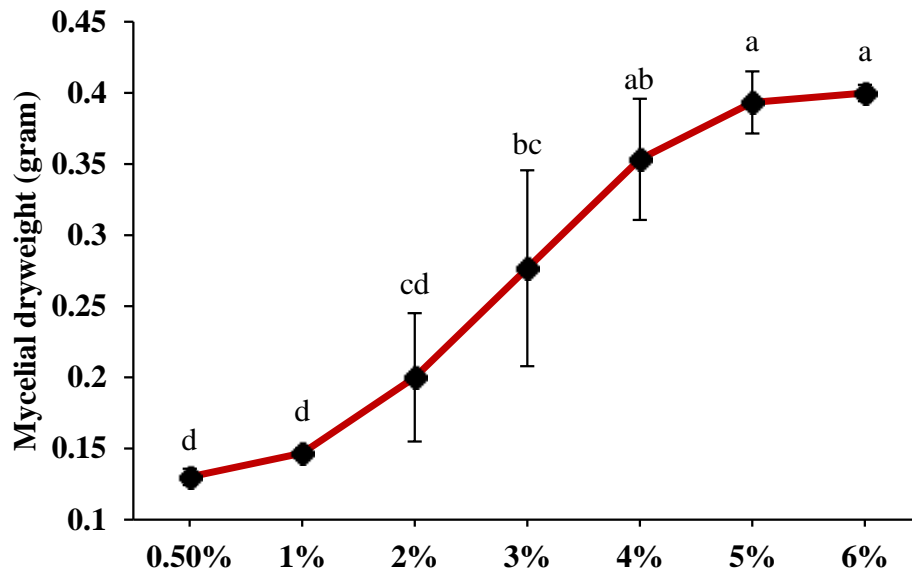


Figure 7. Effect of varying concentration of RP on mycelial dry weight (data are means of three replicates \pm SE). Means with the same alphabets are not significantly different at $p = 0.05$ (Duncan's).

pH was observed at 6% sodium chloride (2.80 ± 0.03) followed by 5% (3.04 ± 0.07) and 3% (3.22 ± 0.04) (Figure 8). Initially an increase in fungal biomass was observed upto 1% sodium chloride concentration and then decreased with the increasing level of sodium chloride (Figure 9) which is significant at the level of $p < 0.001$.

DISCUSSION

In agreement with several published reports, this study confirms phosphate solubilizing efficiency of a soil fungus. A number of different filamentous fungi have been reported as potential P solubilizers by many researchers

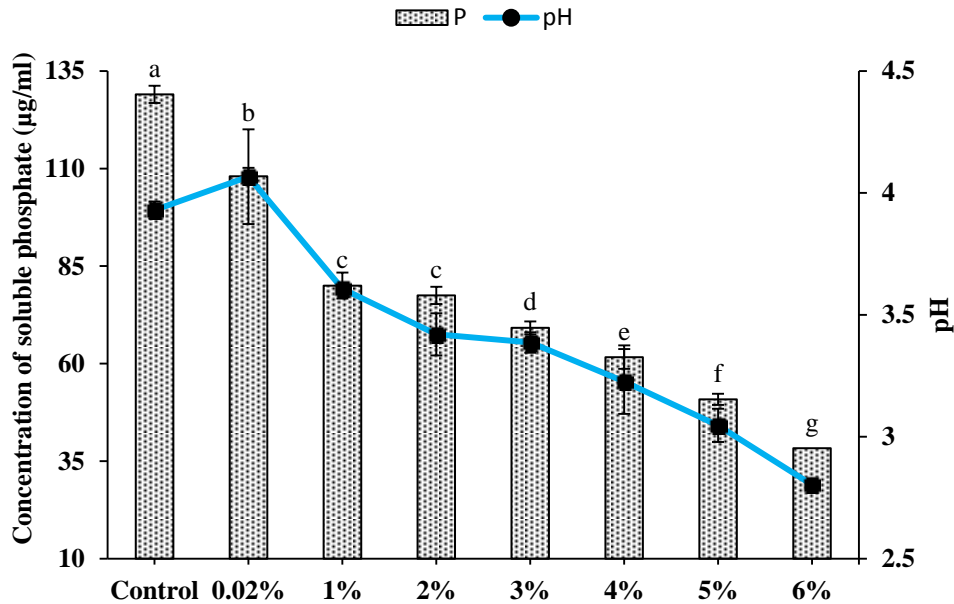


Figure 8. Effect of varying concentration of NaCl on concentration of soluble P and pH of the culture medium (data are means of three replicates \pm SE). Means with the same alphabets are not significantly different at $p = 0.05$ (Duncan's).

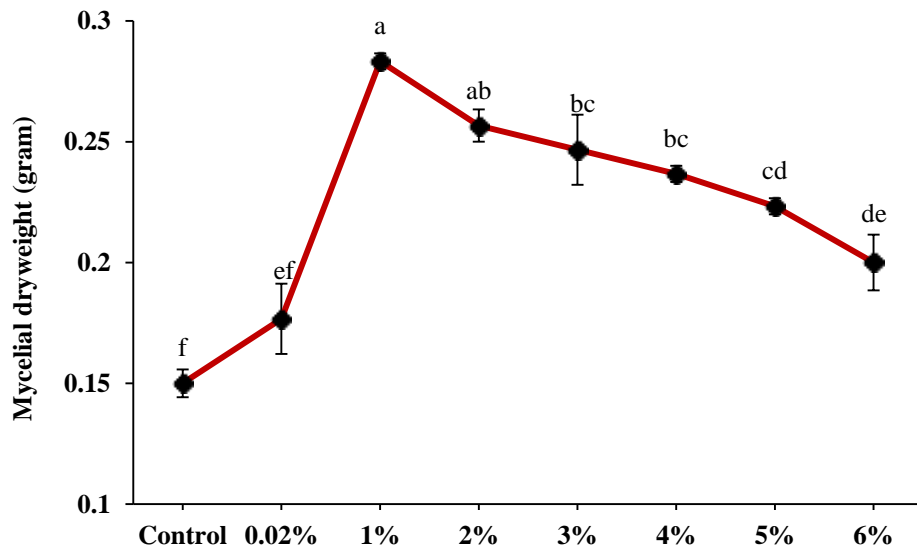


Figure 9. Effect of varying concentration of NaCl on mycelial dry weight (data are means of three replicates \pm SE). Means with the same alphabets are not significantly different at $p = 0.05$ (Duncan's).

and among these fungi, *Aspergillus* and *Penicillium* are predominant (Mahamuni et al., 2012; Posada et al., 2013). *Penicillium pupurogenum* has been reported as phosphate solubilizer by earlier researchers (Scervino et al., 2010; Yadav and Tarafdar, 2011).

The appearance of clear halozone around the colony indicated phosphate solubilization by the fungus which is

in accordance with the findings of Gupta et al. (2007). The SI value for the fungus was found to be 2.29. Similarly, Seshadri et al. (2004) reported the SI values for ten different strains of *Aspergillus niger* that ranged from 1.2-3.4 on the fifth day of incubation. In another study, the SI for *P. pupurogenum* was found to be 1.3 after four days of incubation (Yasser et al., 2014). The RP was

poorly solubilized as compared to TCP. The most likely explanation is that the RP is more complex in structure than TCP. In addition to containing P-Ca, the RP contain other elements such as Mn, Zn, Fe and Cu which certainly affect organic acid production even in low concentration (Gadd, 1999; Mendes et al., 2014). Our data showed reduction in pH of the medium. Several earlier studies have reported that P solubilization is associated with the production of organic acids, which chelate cations through their carboxylic acid group and convert it into soluble form (Kpombrekou and Tabatabai, 1994; Sane and Mehta, 2015). However, with further incubation, phosphate solubilization increased with increase in pH. These results support earlier reports that phosphate solubilization is not necessarily associated with organic acid production (Abd Alla, 1994; Nautiyal et al., 2000). Cause of increase of pH of the medium after a certain period can be explained as the catabolic activity of fungi on organic acid as reported by Nielsen et al. (1994).

Different fungi use different carbon sources, and based on the carbon source, fungi use alternative metabolic pathways to produce organic acids. The process of P solubilization is greatly influenced by the organic acids produced in the medium because it provides a source of protons for solubilization (Gadd, 1999). The organic acids are the product of the microbial metabolism; mostly by oxidative respiration or by fermentation of organic carbon sources (For example, glucose) (Atlas and Bartha, 1997). Such organic acids can either directly dissolve the mineral P as a result of anion exchange or by chelating cations associated with P (Omar, 1998). It has been also reported that the nature of acid produced is more important than the quantity of the acid (Srividya et al., 2009). Glucose and xylose was found to be the best source of energy for the fungi (Rose, 1957). *Penicillium citrinum* Thom. showed maximum significant P solubilization in the presence of glucose followed by glycerol, maltose and sucrose. Drop in pH was also recorded for all the tested carbon sources. Similar results were reported by many researchers for various other fungi (Yadav et al., 2011; Bhattacharya et al., 2015). The maximum wet fungal biomass of *A. niger* was observed in the presence of mannitol (Seshadri et al., 2004).

For many fungi, NH_4^+ driven proton release seems to be the sole mechanism to promote P solubilization (Illmer and Schinner, 1992). Further, Reyes et al. (1999) reported the involvement of the H^+ pump mechanism in the solubilization of small amounts of P in *Penicillium rugulosum*. Ammonium sulphate was found to be the best source of nitrogen for the solubilization of P. Similar results have been highlighted by earlier researchers (Pradhan and Shukla, 2005; Srividya et al., 2009). Increase in the level of soluble P was recorded in medium supplemented with ammonical nitrogen as nitrogen source by *Penicillium bilaji* (Asea et al., 1988). However, any significant relationship between the level of

soluble P and pH drop could not be found. This indicates that acid production is not the only reason for P solubilization. Yeast extract was reported as the best nitrogen source utilized by *P. chrysogenum* for maximum phosphate solubilization (El-Badry et al., 2015). Though, the higher P solubilization was observed in the case of control, lowest fungal mycelial dry weight was recorded in control as compared to other nitrogen sources. As the amount of fungal biomass continued to increase slowly, the determined phosphate in the solution probably corresponded to the amount which was not consumed by the mycelium (Vassilev et al., 1996). Thus, the nature of carbon and nitrogen source used by the microbes directly influence the organic acid production and thereby the solubilization activity.

The highest level of P solubilization was found at 0.5% RP concentration, the value decreases with increase in RP concentration. *A. niger* (PSF4) showed highest soluble P content at 1% RP concentration. Also, the greater conversion of RP into soluble P was observed in the presence of lower concentration of RP. A sharp decline in the soluble P content was recorded with increasing concentration of RP (Panda et al., 2008). This may be due to the inhibitory effect of metals like Al, Ca and Fe present in RP. These metals inhibit the growth and activity of fungi or cause change in pH of the medium, which in turn affect P solubilization (Gaur and Sacher, 1980). Xiao et al. (2008) also reported a decrease in soluble P content when concentration of RP increased from 2.5 to 4.0 g L^{-1} by *Penicillium expansum*, *Mucor ramosissimus* and *Candida krissi*. However, increase in mycelial dry weight was observed with increase in RP concentration. Under limited P availability (when the very high P fixing soils were present), *Mortierella* sp. tend to accumulate P in its cells instead of releasing it into the growth medium (Osorio et al., 2015).

A simultaneous decrease in the solubilization of P and mycelial dry weight were recorded with increasing concentration of sodium chloride. Similar finding was reported by many workers (Rosado et al., 1998; Nautiyal, 1999; Kang et al., 2002; Saber et al., 2009). Xiao et al. (2011) tested the P-solubilizing efficiency of *A. niger*, *A. japonicus* and *Penicillium simplicissium* in the presence of 0% to 3.5% NaCl concentration. They observed an increase in the level of soluble P and the growth of fungal spores when the NaCl concentration was increased from 0 to 1.0%. But, a gradual decrease in both the parameters was observed above 1.0%. The reduction in P activity under high salt environment can be explained by the adverse effect of salts in growth and cell proliferation resulting in a loss of solubilization efficiency and sequestration or neutralization of protons or acids by chloride ions resulting in a reduction of solubilization activity. Also, the decrease in microbial population with increasing concentration of NaCl can be attributed to decrease in cytoplasmic water activity of the microbes caused by the exposure of organisms to the conditions of

hyper osmolarity (Mussarat and Khan, 2014).

Conclusion

These findings clearly indicated that for higher P solubilization, optimization of process parameter is required. Phosphate solubilization and growth were found to be highly influenced by both carbon and nitrogen sources. Further, solubilization of P was influenced by varying the initial concentration of RP. In addition, a significant P solubilization was also observed in different saline concentration. One factor at a time (OFAT) offers the possible finding of the parameters for optimization process, though it is not of first priority in the examination of the interaction between the variables. The fungal strain *Penicillium purpurogenum* Stoll could also be helpful in maintaining the available P in saline soil and its application as biofertilizer could be of great advantage in RP amended soil.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Screening of biologically active constituents from leaves of *Aloe elegans* and their antimicrobial activities against clinical pathogens

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The flourishing of the war b/n pathogens and antibiotics is contingent on the diligent trial to find effective antibiotics; so that to lessen the fear faced by resistant microbes. The screening carried out manifested the presence of saponins, glycosides, tannins, reduced sugars, terpenoids, flavonoids and phenols. Alkaloids and steroids were not traced from any of the extracts. Aqueous and solvent extracts from leaves of *Aloe elegans* were inspected for antimicrobial activity against pathogens namely *Klebsiella pneumonia*, *Salmonella thypharium*, *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* by well and disc diffusion method. Disk diffusion method showed a better result than well diffusion method. The highest poisoning against all the microbes was seen by ethanol extract. The extract showed unsurpassed antifungal activity than antibacterial activity. Minimum inhibitory concentration (MIC) assay was ascertained for this extract against bacteria and fungi. Ethanol extract bespoke maximum toxicity against *S. aureus* at 0.78 mg/ml concentration succeeded by *E. coli* at 1.56 mg/ml concentration. The upshots provide justification for the possibility to utilize plant extracts to treat sundry infectious diseases.

Key words: *Aloe elegans*, minimum inhibitory concentration (MIC), antimicrobial, phytochemicals.

INTRODUCTION

Plant materials remain a crucial source to fight stringent diseases in the world. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds (Edeoga et al., 2005). The widespread use of a limited number of antimicrobial agents concomitantly with the reduced

arsenal of drugs with antimicrobial function, has led to the development of resistance to drugs that oppose both fungal and bacterial infections, which has been an increasing problem (Zida et al., 2016). The medicinal plants are useful for healing as well as for curing of human diseases because of the presence of

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phytochemical constituents (Nostro et al., 2000). They are naturally occurring in the medicinal plants, leaves, and roots that have defense mechanisms and protect against various pathogens. Phytochemicals are primary and secondary compounds. Chlorophyll, proteins and common sugars are included in primary constituents and secondary compounds have terpenoids, alkaloids, and phenolic compounds (Krishnaiah et al., 2007). terpenoids exhibit various important pharmacological activities, that is, anti-inflammatory, anti-cancer, anti-malarial, inhibition of cholesterol synthesis, anti-viral and anti-bacterial activities (Mahato and Sen, 1997). Alkaloids are used as anesthetic agents and are found in medicinal plants (Herouart et al., 1988). Ergo, it is momentous to take action for the screening of different plants by considering their different floral parts in order to affirm their use as medicinal valued material and to bespeak the active principles by compartmentalization and characterisation of their constituents. In our investigation of *Aloe elegans*, an aqueous extract and three solvent extracts of leaves of *A. elegans* were screened for their phytochemical constituents and their possible antimicrobial activity.

Antimicrobial study of plants has gained prevalence and many researchers (Anzabi, 2014; Mariana et al., 2017) have shown that plants could be used as alternative medicines to combat the problem presented by resistant pathogens. *Aloe vera*, one of the species of Aloes, has been well studied in every aspect, it could be harnessed and has been used in the cosmetic industry. The successful and many uses of *A. vera* has propelled researchers to explore other plants with rigours. *Aloe elegans* is a regionally endemic species of the Aloeacea family which is only found in Eritrea and Ethiopia (Demissew and Nordal, 2010). To the best of our knowledge for this species, few or incomplete studies have been carried out in the struggle to find an alternative drug from plants. The present study investigated the potential use of *A. elegans* as a better source of antimicrobial agents.

Aloe species have been used for a long time as folk medicine for the treatment of constipation, burns and dermatitis. Recently, some species of *Aloe*, have been used in a wide range of skin and hair care products, and also form the basis of health drinks and tonics. The slimy gel inside the leaves consists of a complex mixture of polysaccharides, amino acids, minerals, trace element and other biologically active substances, such as enzymes. *Aloe* species have been the source of laxative drugs, the main purgative principle being an anthrone-C glycoside, *aloin*, which occurs at levels of between 18 and 30% of the dried product (British Pharmacopoeia, 1993, United States Pharmacopoeia, 1995). Recent research has indicated that aloe might kill the bacteria responsible for tuberculosis, *Mycobacterium tuberculosis*, and also the herpes virus causing herpes genitalis. Research has further shown that aloe inhibits growth of many common organisms such as yeasts, fungi, and

bacteria infecting wounds (Demissew and Nordal, 2010).

MATERIALS AND METHODS

Plant preparation

A. elegans leaves were gleaned from subzone of Mai-Nefhi, 20 km north east of Asmara. *Aloe elegans* was authenticated by a taxonomist in the Department of Botany, College of Mai-Nefhi, Prof. Gebrehiwet Medhanie. The plant material was washed to remove dirt and adhering materials and was sliced. The gel was obtained simply by rubbing using knife, and then dried in a shed. After being dried in a shade, the plant materials were ground in electric grinder. The powder was packed in plastic bag until further use.

Preparation of extracts

For water extraction, Parekh and Chanda (2006) method was followed with little modification. Air dried leaves of *A. elegans*, which was packed in plastic bag as a powder was summed to 500 ml of distilled water. This mixture was boiled with very low heat for about 120 min. After that it was cooled and filtered through 8 layers of muslin cloth for a sterility purpose, and finally centrifuged at 5000 g for 10 min and the supernatant was gleaned. This was done twice. Lastly, the supernatant was concentrated to make the final volume, one fourth of the original volume using a rotavapour and the crude extract was dissolved in distilled water and stored at 4°C for further usage (Parekh and Chanda, 2006).

For solvent extraction, Parekh and Chanda (2006) method was followed with some little modification. Air dried leaves of *A. elegans* (100 g), which was packed in plastic bag as a powder; was mixed with 500 ml of organic solvent (petroleum ether, ethyl acetate and ethanol) in 500 ml flask with cotton wool as a lid and then kept on rotary shaker at 220 rpm for 72 h. Succeeded by collecting the supernatant and filtered through 8 layers of muslin cloth for a sterility purpose and finally the solvent was evaporated to make the final volume one fourth of the original volume using a rotavapour and the crude extract was dissolved in dimethylsulfoxide (DMSO) and stored at 4°C (Parekh and Chanda, 2006).

Qualitative phytochemical screening

Phytochemical screening by standard methods were implemented to the newly groomed extracts to assure the absence or the presence of the proceeding phytochemicals such as tannins, steroids, alkaloids, glycosides, phenols, reduced sugars, alkaloids, saponins and flavonoids (Parekh and Chanda, 2006).

Test microorganisms

Microorganisms were given by National Health Laboratory of Eritrea. Two gram-positive bacteria: *Bacillus subtilis* and *Staphylococcus aureus*; three gram-negative bacteria: *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Escherichia coli* and a fungi: *Candida albicans* were investigated.

Preparation of inoculum

Some colonies from nutrient agar of pure growth of each tested organism were transferred to 5 ml of Muller Hinton Broth for bacteria and Sabouraud dextrose broth for fungi. Both broths were incubated overnight at 37°C. The suspension was diluted with

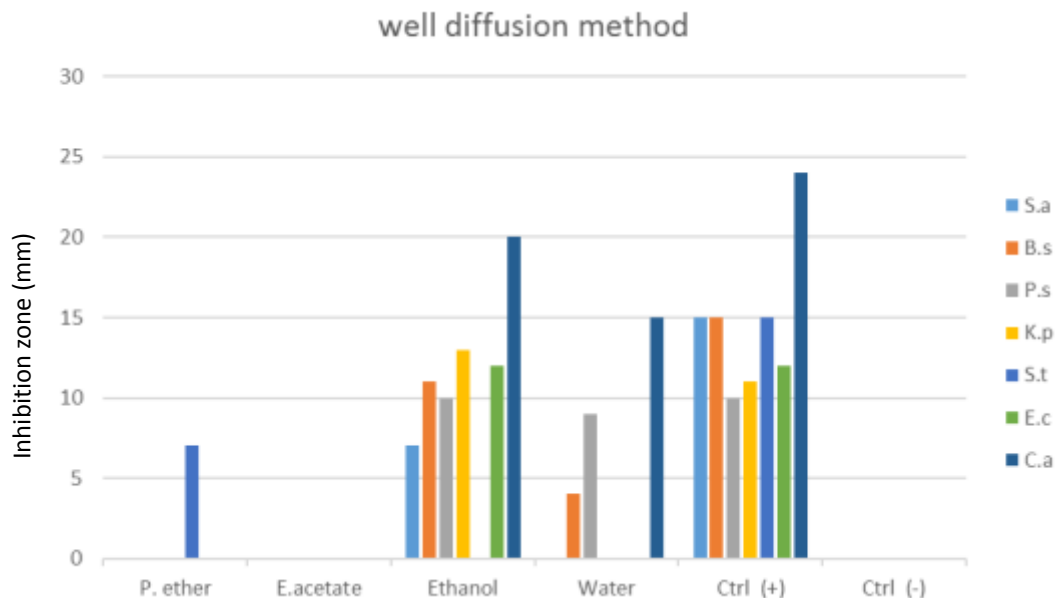


Figure 1. The results for well diffusion method.

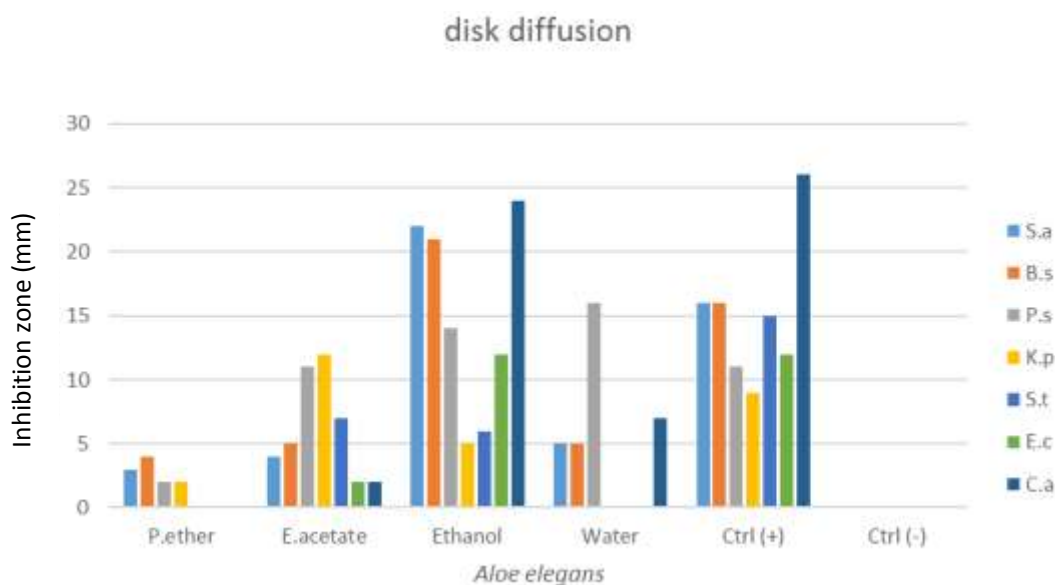


Figure 2. Results for the results of disk diffusion method.

sterile distilled water to obtain approximately 106 to 108 CFU/ml (McFarland, 1907).

Preparation standard concentrations

One gram of each solvent and aqueous extract were dissolved into 5 ml of dimethylsulphoxide and 5 g of sterilized water, respectively. This harvested in 200 mg/ml of stock solution as a standard concentration of both types of extracts. Solvent extracts were pasteurized at 62°C while water extract was sterilized by using membrane filters.

Antimicrobial assay

The antimicrobial assay was implemented by dual procedures, namely, disk and well diffusion methods (Bauer et al., 1966; Perez et al., 1990). A 100 µl of inoculum (108 CFU) was inoculated with the Muller Hinton Agar and was made to be poured into sterile petri plates (HiMedia).

The well diffusion procedure was done by pipetting 100 µl of our test compound into the wells; which was built by sterilized borer of 0.6 cm. Similarly, the diffusion procedure was performed by pipetting our experiment compound (100 µl) onto the disc (0.6 cm) which was stuck in the agar. The plates were allowed to be

Table 1. The phytochemicals of *Aloe elegans*.

Phytochemicals	Solvent extracts			
	Petroleum ether	Ethyl acetate	Ethanol	Distilled water
Flavonoids	-	-	-	+
Glycosides	-	+	-	-
Phenols	-	-	+	-
Reduced sugars	-	-	-	-
Saponins	+	+	+	+
Tannins	-	-	+	-
Terpenoids	-	-	+	-
Alkaloids	-	-	-	-
Steroids	-	-	-	-

Presence of phytochemicals = "+", Absence of phytochemicals = "-".

incubated for 24 h at 37°C. Gentamycin (10 µg/disc) and nystatin discs (100 µg/disc) were used as positive controls for the bacteria and fungus, respectively. Negative control was prepared using respective solvent. Microbial growth was determined by measuring the diameter of zone of inhibition. The experiment was done in triplicate form and the mean values with their standard deviation are presented.

Minimum inhibitory concentration (MIC) assay

MIC procedure was exercised to an extract which was efficient in inhibiting the pathogens by both the well and disk diffusion method. MIC of ethanol extract was ascertained by INT calorimetric assay (a rapid p-Iodnitrotetrazolium chloride) (Eloff, 1998). Concisely, the test solvent sample was dissolved in DMSO which in turn was dissolved in MHB. The yielded solution was diluted serially by two fold in 96 well microtiter plate. An inoculum of 100 µl, which was calibrated to 1.5×10⁶ CFU/ml groomed in MHB which was finally added. Plates with inoculums were sealed with a sterilized lid and mixed with a shaker to blend the composition, and was allowed to be incubated at 37°C for about 18 h. The amount of DMSO was made to be <2.5%, which means DMSO could not make an impact to the growth of microorganisms. A negative control was experimented parallel to the experiment, which contained a well with a 100 µl of inoculum+MHB+DMSO. Gentamycin was taken as a reference. After 18 h of incubation with 37°C, proceeded by addition of 40 µl INT (0.2 mg/ml), and incubated at 37°C for 30 min. The MIC of the ethanol extract was disclosed. Those microbials which were viable changed this yellow dye to pink. MIC is defined as it is the lowest concentration that precludes this mutability and results complete inhibition of the growth of microbes.

RESULTS AND DISCUSSION

Phytochemical constituents such as phenols, flavonoids, tannins, alkaloids, saponins and many other aromatic compounds are secondary metabolites of plants that serve a defence mechanism against predation by many microorganisms, insects and other herbivores (Bonjar et al., 2004). The phytochemical result is depicted in Table 1. The present study evinced the presence of medicinally active constituents of saponins, glycosides, tannins, terpenoids, flavonoids and phenols. Steroids and

alkaloids were not present in the plant extracts studied. It is not surprising that ethanol extract of *A. elegans* showed significant inhibition of all tested bacterial and fungal organisms. Almost all the phytochemicals that fight against microbes have been contained in the ethanol extract. The bioactive compounds that are present in plants act by peculiar mechanism and exert antimicrobial activity. The antimicrobial activity of the extract may be due to the presence of phenols, tannins, terpenoids, and flavonoids. Phenols and tannins act their antimicrobial activity by binding to adhesins, enzyme inhibition, substrate deprivation, complex with cell wall, membrane disruption, and metal complexion. Terpenoids act their antimicrobial activity by membrane disruption. Flavonoids act in a way by binding to adhesins and complexing to cell wall of the microbes (Prashant et al., 2011). Next to ethanol extract, water extract showed an inhibition which cannot be underestimated; this might be due to the presence of flavonoids.

Out of the four extracts tested, ethanol extract was the best inhibitor of microbes. It showed inhibition to all organisms followed by an aqueous extract in both methods. The outcome for the antimicrobial results is depicted in Tables 2 and 3 as well as Figures 1 and 2, respectively. In this study, disk diffusion showed better inhibition than well diffusion method. The reason could be due to the plant extract added, which was able to diffuse in the bottom of the plate and thus be far from bacteria grown on the surface. Petroleum ether and ethyl acetate were the weakest extracts to show minimum inhibition in this study which might be caused by being weak solvents to dissolve the active components of the plant. Out of the five bacteria, *S. aureus* was the most susceptible organism which inhibited up to 22 mm, whilst *Salmonella thypharium* and *E. coli* proved to be the most resistant strain. Antifungal result was very promising; it showed up to 24 mm zone of inhibition in ethanol extract. The positive control for fungus showed its highest inhibition zone of up to 26 mm. Further study should be done to determine what would be their combined effect. In

Table 2. Well diffusion method.

Extract	Microorganism (zone of inhibition (mm in diameter) (Mean \pm SD) (n=3))						
	S.a	B.s	P.s	K.p	S.t	E.c	C.a
P.ether	-	-	-	-	7 \pm 0.25	-	-
E.acetate	-	-	-	-	-	-	-
Ethanol	7 \pm 0.28	11 \pm 0.78	10 \pm 0.25	13 \pm 0.90	-	12 \pm 0.58	20 \pm 0.21
Water	-	4 \pm 0.42	9 \pm 0.82	-	-	-	15 \pm 0.12
Ctrl (+)	15 \pm 0.36	15 \pm 0.12	10 \pm 0.22	11 \pm 0.10	15 \pm 0.11	12 \pm 0.12	24 \pm 0.24
Ctrl (-)	-	-	-	-	-	-	-

Escherichia coli (E.c), *Klebsiella pneumonia* (K.p), *Pseudomonas aeruginosa* (P.s), *Salmonella thypharium* (S.a), *Bacillus subtilis* (B.s), *Candida albicans* (C.a), Control(ctrl.) ; diameter of inhibition zone less than 6mm is considered as negative(-). (+) control= gentamycin (10 μ g/disc) for bacteria and nystatin discs (100 μ g/disk), (-) control= DMSO for the solvent extracts and Distilled water for the aqueous extract.

Table 3. Disk diffusion method.

Extract	Microorganism (zone of inhibition (mm in diameter) (Mean \pm SD) (n=3))						
	S.a	B.s	P.s	K.p	S.t	E.c	C.a
P.ether	3 \pm 0.06	4 \pm 0.08	2 \pm 0.09	2 \pm 0.21	-	-	-
E.acetate	4 \pm 0.22	5 \pm 0.21	11 \pm 0.12	12 \pm 0.05	7 \pm 0.23	2 \pm 0.50	2 \pm 0.23
Ethanol	22 \pm 0.36	21 \pm 1.0	14 \pm 1.0	5 \pm 0.85	6 \pm 0.91	12 \pm 0.89	24 \pm 0.20
Water	5 \pm 3.6	5 \pm 0.88	16 \pm 0.06	-	-	-	7 \pm 0.56
Ctrl (+)	16 \pm 0.36	16 \pm 0.12	11 \pm 0.22	9 \pm 0.10	15 \pm 0.11	12 \pm 0.23	26 \pm 0.8
Ctrl (-)	-	-	-	-	-	-	-

Escherichia coli (E.c), *Klebsiella pneumonia* (K.p), *Pseudomonas aeruginosa* (P.s), *Salmonella thypharium* (S.a), *Bacillus subtilis* (B.s), *Candida albicans* (C.a), Control(ctrl.) ; diameter of inhibition zone less than 6mm is considered as negative(-). (+) control= gentamycin (10 μ g/disc) for bacteria and nystatin discs (100 μ g/disk), (-) control= DMSO for the solvent extracts and Distilled water for the aqueous extract.

Table 4. Minimum Inhibitory Concentration of *A. elegans*.

Microorganism	Efficacious ethanolic extract	
	<i>A. elegans</i> (mg/ml)	Gentamycin/Nystatin (μ g/ml)
<i>E. coli</i>	1.56	12.5
<i>K. pneumonia</i>	12.5	100
<i>P. aeruginosa</i>	6.25	1.5
<i>S. thypharium</i>	12.5	100
<i>S. aureus</i>	0.78	1.5
<i>B. subtilis</i>	25	12
<i>C. albicans</i>	6.25	1.5

(+) control= gentamycin for bacteria and nystatin for fungus.

general, Gram negative bacteria were resistant strains compared to gram positive strains; the reason is simple; lipopolysaccharide (LPS) layer of gram-negative bacteria in outer membrane have a high hydrophobicity which acts as a strong permeability barrier against hydrophobic molecules. Hydrophobic molecules can pass through cell wall of gram positive bacteria easier than the gram-

negative bacteria, because cell wall of the gram-positive bacteria contained only peptidoglycan (Ababutain, 2011).

Ethanol extract showed the highest activity against pathogens which ultimately became the reason to be chosen for MIC test. Outcomes for MIC are shown in Table 4. Ethanol extract showed maximum toxicity against *S. aureus* at 0.78 mg/ml concentration which is

succeeded by *E. coli* at 1.56 mg/ml. Poor toxicity was seen against *B. subtilis* at 25 mg/ml.

Conclusion

The upshots provides justification for the use of ethanol extract as a medicine to treat sundry infectious diseases and *A. elegans* might be a replacer plant for *A. vera* usage after more researches are done on this important plant.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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