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Isolation and Characterization of Biotechnologically Potent *Micrococcus luteus* Strain From Environment

Ali Akbar,[1,2]* Uzma Sitara,[3] Imran Ali,[4] Niaz Muhammad[5] and Shabir Ahmad Khan[1]

[1]*Department of Microbiology, Faculty of Life Science, University of Balochistan Quetta, 87300, Pakistan*
[2]*Food Engineering and Bioprocess Technology, School of Environment, Resources and Development, Asian Institute of Technology, Klong Luang, Pathumthani 12120, Thailand*
[3]*Food Quality Storage Research Institute, Southern-Zone Agricultural Research Centre, Pakistan Agricultural Research Council, Karachi-75270, Pakistan*
[4]*Plant Biomass Utilization Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand*
[5]*Institute of Soil and Water Resources and Environmental Sciences, Zhejiang Provincial Key Laboratory of Subtropical Soil and Plant Nutrition, Zhejiang University, Hangzhou 310056, China*

Abstract.- This study was conducted to isolate antagonistic and biotechnologically potent bacteria from environment. *Micrococcus luteus* was the only bacterium found producing antimicrobial metabolites and exhibited good probiotic characteristics. *Micrococcus luteus* showed antibacterial activity against foodborne pathogens, *Salmonella typhimurium* (16-20 mm), *Listeria monocytogenes* (11-15 mm) and *Escherichia coli* (5-10 mm). The bacterium was also found active against *Klebsiella pneumoniae* and *Staphylococcus aureus*. It was found producing industrially important enzymes including protease, lipase and phytase, confirmed by screening with specific substrate supplementations. The bacterium was able to survive in 2% bile salt and 4% NaCl with good probiotic potential. The DPPH radical scavenging activity of the *Micrococcus luteus* was found 79.61±0.02%, while the total phenolic content was 200µg/mL equivalent to gallic acid.

Keywords: Probiotics, industrial enzymes, protease, lipase, phytase, food safety, *Micrococcus luteus, Klebsiella pneumoniae, Staphylococcus aureus.*

INTRODUCTION

Infectious diseases related to bacterial pathogens remains a big problem in developed and developing countries (Mirnejad *et al*., 2013). The increasing antibacterial drug resistance to commonly in-use antibiotics is enhancing the severity of infections (Jamalifar *et al*., 2011), and posing substantial burden over health and economy along with increase in mortality and morbidity (Tanvir *et al*., 2012; Akbar and Anal, 2011). The bacteria with probiotics and antagonistic properties are proved capable of reducing the microbial load in food and showed *in-vitro* and *in-vivo* activities against pathogens (Akbar and Anal, 2014a; Mirnejad *et al*., 2013).

Alternative sources for pathogens control, such as probiotics, antioxidant, antimicrobial plant materials and nanoparticles etc are getting appraisal due to its wide range of acceptability for human use (Akbar and Anal, 2014b; Abd El-Rhman *et al*., 2009). Exploring new and existing microbial resources for its novel uses are of utmost important (Malik *et al*., 2013; Akbar and Anal, 2011). Some groups of microbes such as lactic acid bacteria and *Bifedobacterium* are known having: probiotic capability, application in food safety and food enrichment are repeatedly reported (Olorunfemi *et al*., 2006), whereas member of other groups such as, *Leuconostoc* and yeast are also important due to its human friendly activities (Ibrahem, 2013). Bacteria belonging to pathogenic and opportunistic pathogenic groups, rarely known as probiotics such as, *Pseudomonas* and *Micrococcus* spp. are also reported with its antibacterial capability against some other bacteria (Abd El-Rhman *et al*., 2009).

The *Micrococcus* spp. is commonly known as normal skin microflora and its antibacterial activity has rarely been reported against different pathogens. This bacterium is ubiquitous in nature and can be

* Corresponding author: aliakbar.uob@gmail.com
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easily found in natural environments (Willey *et al*., 2008). It can degrade hydrocarbons (Zhuang, 2003), toxic organic pollutants and can tolerate the presence of metals (Sandrin and Maier, 2003). Its property of precipitating gold by crystallizing it on their surface can be used for gold enrichment in low concentration gold ores (Marakushev, 1991). This organism can be exploited for its abilities of producing technologically important compounds and processing for industrial applications. The data regarding antibacterial, probiotic activities and industrially important metabolites production of this bacterium is limited and it need further explorations. Determination of such activities from an environmental isolates of *Micrococcus luteus* is the focus of this study. The objective of this study was to isolate environmental bacteria with antibacterial capability against some target bacteria, and to characterize and identify its possible role for the welfare of human being in term of its probiotic capability or production of industrially valuable compounds.

MATERIALS AND METHODS

Isolation and identification

Environmental sampling of open (agriculture fields and garden) and closed (room, kitchen and laboratory) environments was conducted for isolation of bacterial species with antimicrobial activity against pathogenic bacteria. The colonies with similar morphology on the sampling plate were considered as duplicate and only prominent and isolated colonies were sub-cultured for identification.

Sampling plates (50) each containing media deMan Rogosa and Sharpe (MRS) agar (Himedia, India) and nutrient agar (Merck, Germany) were exposed randomly to the environment for 1 min at 10 different places in different time interval in order to obtain the environmental bacteria. The plates were observed for the presence of visible and distinguished bacterial colonies after 24-48 h aerobic incubation at 37°C. The fungal colonies were ignored and only bacterial colonies were sub-cultured on MRS agar for antagonistic activity determination. The bacteria with antibacterial activity were identified with the help of Gram's staining, colonies characteristics, catalase, oxidase, coagulase reactions, bacitracin susceptibility and biochemical profiling using API kits (Biomerieux, France).

Antibacterial activity determination

The antibacterial activity of the selected isolates was evaluated with the help of three different methods including, agar well diffusion, spot on lawn and supernatant mix methods following (Akbar and Anal, 2014a).

In agar well diffusion method MRS broth cultured (24 h) with selected bacteria were centrifuged at 8,000 rpm for 20 min, the supernatant were filtered (0.2 µm pore diameter, Minisart, Germany) sterilized and 100 µL were poured in 6 mm wells previously made on Muller Hinton agar (Merck, Germany) seeded with the target bacteria and incubated at 37°C for 16-24 h.

In spot on lawn method 5 µL of the selected bacteria were spotted on the surface of MRS agar and incubated at 37°C for 24 h. A sterile semisolid nutrient agar (0.7 % agar w/v) seeded with the target bacteria were poured over the surface of visible colonies of selected bacteria in MRS agar and incubated at 37°C for 16-24 h. Clear zone around the colonies were observed and recorded.

In supernatant mix method, an equal amount of filtered sterile supernatant from 24 h MRS culture was mixed with a double strength nutrient broth. Target bacteria (10^6 CFU/mL) were inoculated to the test tubes. Nutrient broth containing test bacteria but no supernatant was used as positive control. The tubes were incubated at 37°C for 24 h and the growth of target bacteria in the tubes was determined with the help of standard plate count and optical density measurement at OD_{600} using spectrophotometer.

Determination of total phenolic content

Total phenolic contents were determined following the methodology used by Arora and Chandra (2010). Test samples (0.5 mL) were mixed with 0.2 mL of Folin–Ciocalteu (FC) reagent and incubated at room temperature. After 10 min, an amount (0.6 mL) of 20% (w/v) sodium carbonate was added to the tubes. The reaction mixture was incubated at 40°C for 30 min. The optical density

was determined at absorbance 765 nm for phenolic activity. All the tubes were properly protected from light by covering with aluminium foil. Gallic acid was used as standard.

Determination of DPPH radical-scavenging activity

The antioxidant activity against 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical was carried out by mixing DPPH methanol solution (0.025g DPPH/1000 mL methanol) with 0.5 mL of supernatant (24 h culture) and kept for 30 min in dark place at room temperature. The absorbance was measured at 517 nm. Methanol was used as a blank, while DPPH methanol solution without sample was used as control. Following formula was used for scavenging activity calculation (Pianpumepong and Noomhorm, 2010).

Scavenging activity (%) = [1-(absorbance of sample/absorbance of control)] x 100

Determination of probiotics characteristic

The bacteria with a broad spectrum of antibacterial activity in all three type of antibacterial test were further selected for its probiotic properties and biotechnologically important products screening. The probiotics characteristics of the bacteria were determined by exposing it to different growth conditions following the methodology of (Pianpumepong and Noomhorm (2010) and Kaushik *et al.* (2009) with slight modifications.

Acidic and alkaline pH tolerance

Fresh culture of test bacteria 2 µL (10^7-10^8 CFU/mL) was inoculated to MRS broth with a pH adjusted at 2.0, 3.0, 5.0, 8.0 and 10 priorly with 2 M HCl / NaOH and incubated at 37°C for 24 h. Same bacteria in normal MRS broth were used as a control. Bacterial growth was confirmed with the help of visible observation and standard plate counting.

Salt tolerance

Fresh culture 2 µL of test bacteria (10^7-10^8 CFU/mL) was introduced to the MRS agar and broth supplemented with 4, 6 and 8 % of salt (NaCl) and incubated at 37 °C for 24 h. Normal MRS broth and agar inoculated with same bacteria was used as control. Visible growth in MRS agar and broth was observed for results documentation.

Bile salt tolerance

Fresh culture 2 µL (10^7-10^8 CFU/mL) was introduced to the MRS agar and broth supplemented with 0.3, 1.0 and 2.0% of bile salt (Oxgall) (Biomark, India) and incubated at 37°C for 24h. Normal MRS broth and agar inoculated with same bacteria was used as control. Visible growth in MRS agar and broth was observed for results documentation.

Growth on different temperature

Growth ability of the test bacteria in a range of temperature (08, 25, 35, 40 and 44°C) was observed by inoculating it to MRS agar and broth and incubated at 37°C for 24 h.

Determination of hydrophobicity

Fresh culture of test bacteria was centrifuged at 8000 rpm for 20 min and washed twice with sterile normal saline. The cells suspension was measured for optical density (OD_{600}) (A^0). Part of suspension (3 mL) were mixed with 1 mL of toluene and blended for 2 min, left standing for 15-20 min to get two separate phases. The optical density (OD_{600}) of the lower aqueous phase (A) was measured. Percentage of cell surface hydrophobicity (% H) was calculated by using the following equation (Pianpumepong and Noomhorm, 2010).

$$H\ (\%) = \frac{(A^0 - A)}{A^0}\ x\ 100$$

Screening of enzyme production (proteases, lipases, amylases and phytase)

Agar supplemented with specific substrate was used for the screening of industrially important enzymes in this study. Methods by Ali *et al.* (2014) were followed with slight modifications.

Nutrient and MRS agar supplemented with 10% skim milk (autoclaved at 115°C for 20 min) was used for the screening of proteases. The bacteria were inoculated to the surface of plates and the plates were observed for clear zone around the bacterial colonies after incubation at 37°C for 24-48 h.

Tween agar (peptone 10 g, calcium chloride hydrate 0.1 g, sodium chloride 5 g, tween-80 10 g, agar 15 g in 1 liter distilled water. pH 7.0-7.4 sterilized at 115°C for 20 min) and Tributyrin agar base (Himedia) supplemented with 1% trybutyrin was used for lipases screening. The agars were inoculated and incubated at 37°C for 24-72 h to observe clear zone around the colonies.

Starch agar (soluble starch 1% in nutrient and MRS agar) was used for the screening of amylase production. The bacteria was inoculated to the Petri dishes and incubated at 37°C for 24-48 h. The plates were stained with iodine and clear zones were observed around the colonies.

The phytase screening medium (PSM) (Glucose 1.5%, $(NH_4)_2SO_4$ 0.5%, KCl 0.05%, $MgSO_4.7H_2O$ 0.01%, NaCl 0.01%, $CaCl_2$. $2H_2O$ 0.01%, $FeSO_4$ 0.001%, $MnSO_4$ 0.001%, sodium phytate 0.5%, agar 1.5%, pH 6.5) was used for the screening of phytase enzyme. The bacteria was inoculated to the plates and incubated at 37°C for 24-72 h. Clear zone around the colonies were observed after incubation for phytase production (Singh *et al.,* 2013).

RESULTS

Twelve different species of bacteria including Gram-negative and Gram-positive were isolated from environment. Out of all 12 bacteria, only one bacterium identified as *M. luteus* was found to have antibacterial activity against a group of foodborne pathogens. The bacteria were initially identified with the help of its morphology; colonies characteristics, biochemical tests using different media, bacitracin test and API Staph ID 32 kit for biochemical profiling. The isolated bacterium (*M. luteus*) was found highly active against the target pathogens including *Salmonella typhimurium, Escherichia coli, Listeria monocytogenes* and showed mild activity against *Staphylococcus aureus* and *Klebsiella pneumoniae* (Fig. 1). No activity was found against *Enterococcus faecalis* (Table I). The antibacterial activity was confirmed by three different methods including agar well, spot on lawn and supernatant mix method. It was observed that *M. luteus* was more active against Gram-negative as compared to Gram-positive bacteria. Increased antibacterial activity of *M. luteus* was observed on MRS agar as compared to nutrient agar. No extensive acidification of nutritional media was observed, only a slight decrease of pH equal to 5 in MRS media were noted at 24 h incubation.

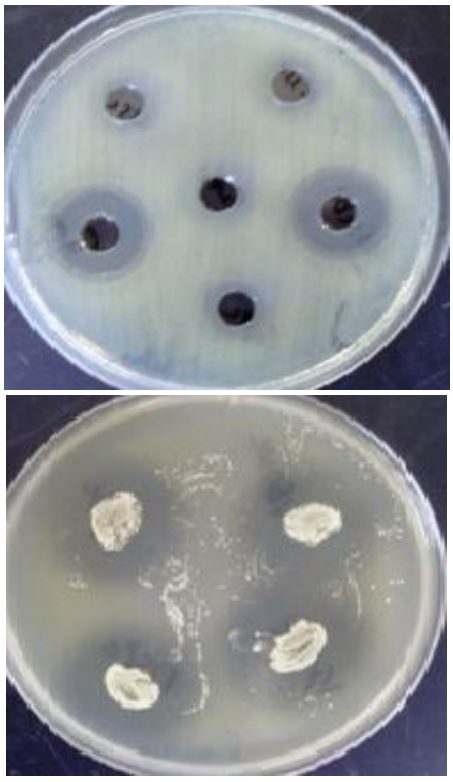

Fig. 1. Antibacterial activities of *Micrococcus luteus* against target bacteria *Salmonella typhimurium* by (A) well diffusion method, (B) spot on lawn method

Table I.- Antibacterial activity of *Micrococcus luteus* against different bacteria.

Target bacteria	Agar well method	Spot on lawn method
Salmonella typhimurium	++++	++++
Escherichia coli	++	++
Listeria monocytogenes	+++	+++
Staphylococcus aureus	+	+
Klebsiella pneumoniae	+	+
Enterococcus faecalis	-ve	-ve

-ve = no inhibition zone, + = 1-5 mm, ++ = 6-10 mm, +++ =11-15 mm, ++++ = 16-20

During the probiotic characteristic analysis, it was observed that isolated *M. luteus* can grow in bile salt (2%) and NaCl 4%, no growth in increasing salt concentrations (6 and 8%) were observed, the bacteria was unable to grow at 8 and 45°C, and in high acidic (pH 3) or alkaline (pH 8) environment. Growth was only observed in medium with pH 5 (Table II). The hydrophobicity of the bacteria to hydrocarbon was found 12±0.19%. The metabolites from *M. luteus* showed 79.61±0.02% DPPH scavenging activity, and total phenolic contents of 200µg/mL equivalent to Gallic acid (Table II). In the enzyme screening phase, the bacterium was found producing protease, phytase and lipase, with inability of amylase production (Fig. 2). Enzyme production was confirmed by observing clear zone around colonies. The bacteria showed good sign of phytase and lipase production after 72 h incubation.

Table II.- Different characteristic of *Micrococcus luteus* isolated from environment.

No	Test	Results
1	Salt tolerance	
	4%	+ve
	6%	-ve
	8%	-ve
2	Bile salt tolerance	
	0.3%	+ve
	0.5%	+ve
	1.0%	+ve
	2.0%	+ve
3	Temperature tolerance	
	8°C	-ve
	25°C	+ve
	30°C	+ve
	40°C	+ve
	45°C	-ve
4	pH tolerance	
	2	-ve
	3	-ve
	5	+ve
	8	-ve
	10	-ve
5	Protease production	+ve
6	Amylase production	-ve
7	Lipase production	+ve
8	Phytase production	+ve
9	Polyphenolic activity	200µg/mL
10	% DPPH activity	79.61 ± 0.02%
11	% Hydrophobicity	12 ± 0.19 %

+ve = Positive, -ve = Negative,

Fig. 2. (A) Protease activity of *Micrococcus luteus* on milk agar, (B) Lipase activity of *Micrococcus luteus* on tween agar

DISCUSSION

Micrococcus is obligate aerobic Gram-positive cocci with the ability to survive in diverse and stress conditions. It was first isolated by Alexander Fleming in 1929 as *Micrococcus lysodeikticus* (Umadevi and Krishnaveni, 2013). Data regarding the antibacterial activity of *M. luteus* is limited and no data regarding its antioxidant and polyphenolic activity are available. Sharma *et al*. (2012) isolated the *Micrococcus* spp. from soil with promising antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Shigella dysenteriae*, *Shigella sonnei*, *Shigella shiga*, *Salmonella typhi* and *Klebsiella Pneumonia*. They also reported xylanases from the same species.

In this study it was noted that *M. luteus* can produce antibacterial and antioxidant compounds in simple nutritional media (nutrient agar/broth).

Increased antibacterial activity was observed, when bacteria was grown in enriched media (MRS). Due to its low acidification activity in the media, this organism can be used against foodborne pathogens and product enrichment, where near to neutral pH is desirable for its quality and physical behaviors, such as milk products.

Abd El-Rhman *et al*. (2009) reported that *M. luteus* isolated from gonads and intestine of Nile tilapia showed antimicrobial activity against *Aeromonas hydrophila* and was found with good probiotic abilities for enhancing the growth of Nile tilapia when utilized as functional diet. Chabrillon *et al*. (2005) reported good probiotic abilities of the genus *Micrococcus* isolated from sea bream, in term of antagonism toward *Vibrio harveyi* and its adherence and competitive exclusion abilities. Irianto and Austin (2003) successfully used (*in-vitro*) *M. luteus* as a probiotic in Rainbow trout, *Oncorhynchus mykiss* farming against *A. salmonicida* infection. Sugita *et al*. (1998) reported the antibacterial activity of *M. luteus* from fish intestine against *Vibrio vulnificus. Micrococcus* was supplemented as diet in fish, having the ability to reduce the pathogenic microbial flora and enhancing food adsorption in the intestine (Abd El-Rhman *et al*., 2009). Umadevi and Krishnaveni (2013) reported the antibacterial activity of *M. lutues* isolated from sea water against *S. aureus*, *Klebsiella* sp. and *Pseudomonas*.

The antioxidant compounds enhance antimicrobial activity due to its toxicity against bacterial cells (Pianpumepong and Noomhorm, 2010). Microorganisms may provide easier way for production of such antioxidants compared to other natural sources such as, plants. Extensive studies are needed to explore the possible beneficial role of this bacterium for the welfare of human beings. Yuen *et al*. (2009) reported efficient proteolytic and lipolytic activities of *M. luteus* isolated from fermented fish sauce. Our results are in agreement with their study. Lawrence *et al*. (1967) and Joseph *et al*. (2011) reported lipases production from *Micrococcus* spp. Phytase are used as food supplement to facilitate the phytate utilization in poultry for phosphate availability, which is an important growth enhancement component in poultry production (Saima *et al*., 2014). Tomova *et al*. (2013) reported protease, lipase and phytase production from *M. luteus* isolated from old cave in Bulgaria. Results of our study are similar with their findings. The *M. lutues* isolated in this study was found biotechnologically important, as it was producing protease, lipase and phytase. It also showed promising results in term of probiotic capabilities, antibacterial and antioxidant metabolites production.

CONCLUSIONS

It was found in this study that *M. luteus* isolated from environment are capable of producing biotechnologically important metabolites. It also produced antimicrobial compounds and was found antagonistic to a group of foodborne pathogens. The bacteria can grow on minimal media with no extra efforts. Thus its use in industrial production of different important metabolites and as a selective probiotic in facilitation of fish and animal farming can be of low cost and result oriented. The bacteria showed promising results in term of its resistance to bile salt and antibacterial activity. This bacterium needs more attention for its exploration to use for human benefits.

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