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Full Length Research Paper

Isolation and screening of antibacterial producing lactic acid bacteria from traditionally fermented drinks ("Ergo" and "Tej") in Gondar town, Northwest Ethiopia

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Traditional fermented beverages that contain lactic acid bacteria have antimicrobial activity for various human pathogens and used for preservation of beverages for a long time. However, there is no scientific report on the antimicrobial activity of lactic acid bacteria isolated from different beverages in Gondar town. The objective of the study was to isolate and screen lactic acid bacteria that show antibacterial activity against clinical and standard human pathogens. Samples of "Ergo" and "Tej" were collected from Arada, Azezo and Kebele 18. A volume of 0.1ml suspension from 10^{-6} dilution of each sample was spread on MRS agar plates and incubated both in aerobic and anaerobic conditions. Morphologically distinct colonies were subcultured to MRS broth and incubated at 37 ^{0}C for 72 hrs in thermostat water bath. The broth culture was centrifuged and the supernatant was used to check the antimicrobial activity against clinical and standard pathogens using agar well diffusion assay. The amount of lactic acid and H_2O_2 produced by the isolates during fermentation was determined using color change after titration. From a total of 18 selected isolates, the cell free supernatant of I_2 and I_3 were able to inhibit the growth of all clinical and standard human pathogens used in this study. The antibacterial activity of the supernatant from I_2 (12.3±1.6 mm to 16±1.7 mm) and I_3 (10±1 mm to 15.7±1.2 mm) were effective compared to Ampicillin (0 to 11±1mm). The maximum amount of lactic acid was recorded at 72 hrs incubation time by both I_2 (1396.24 mg) and I_3 (810.72 mg). Moreover, much amount of H_2O_2 was produced at 72 hrs incubation time by I_2 (3.7 mg) and I_3 (5.35 mg). Thus, isolation and screening of lactic acid bacteria from potential fermented drinks are the sources of antibacterial agents for the treatment of human pathogens. This can give direction for finding antibacterial agent producing microorganisms from fermented drinks use for preservation of foods as well as prevention of health risk problems associated with food borne diseases.

Keywords: Antimicrobial activity, "Ergo", Gondar town, Lactic acid bacteria, "Tej"

INTRODUCTION

Microbial fermentation has played an important role in food processing for thousands of years. It provides a way to preserve food products by increasing their quality as well as safety and reducing the energy required for cooking. These significant changes develop new aroma, flavor, taste and texture that increase the acceptability and the shelf life of the product. Such activities are performed by using different metabolites produced from different microorganisms like lactic acid bacteria (LAB) (Esayas et al., 2008).

Lactic acid bacteria are gram positive, rod or cocci, non-spore forming, catalase- negative bacteria. They are devoid of cytochromes and are often non aerobic habit but are areo- tolerant, fastidious, acid tolerant and strictly fermentative. They are found in different fermented drinks and foods such as sorghum beer, milk, sour dough bread, cassava and pickled (fermented) vegetables (Aslam and Qazi, 2010). They are typically involved in a large number of spontaneous food ferm-

entations such as cheese, yoghurt, butter and kimchi (Lavanya et al., 2011).

Lactic acid bacteria are the most widely used bacteria as starter cultures for the industrial processing of fermented dairy, meat, vegetable and cereal products. Reduction of pH and conversion of sugar to organic acids are the primary preserving actions that these bacteria provide to fermented foods. However, many kinds of foods are still fermented naturally, without the use of starter cultures, by autochthonous lactic acid bacteria, which form characteristics properties of products (Suskovi et al., 2010).

Lactic acid bacteria ferment carbohydrates to lactic acid (the major end product of sugar fermentation for LAB) resulting in a decrease in pH favorable to drinks and food bio-preservation (Tabak et al., 2012). Lactic acid fermentation of drinks and foods also produce antimicrobial agents, such as bacteriocin (Pal et al., 2005; Saranza and Heashengagma, 2011), organic acid, hydrogen peroxide and diacetyl (Saranza and Heashengagma, 2011).

Antimicrobial compounds produced by the lactic acid bacteria can be fully or partially inactivated by proteolytic enzymes (such as chymotrypsim, trypsin, and pepsin) but not affected by lipase, catalase and amylase (Savadogo et al., 2004). The compounds are heat stable up to 102 ^{0}C for 20 min and are active from pH 3 to 10. The highest activity is recorded under acidic condition and the activity is decreased with increasing alkalinity (Abdelbasset and Djamila, 2008).

Lactic acid bacteria such as *Lactobacillus, Pediococcus*, *Streptococcus* and *Leuconostoc* species isolated from traditional Ethiopian fermented beverages show good antagonistic activity against various foods borne pathogens. They prevent and treat diarrhea and clostridium disease. Such antagonistic property is attributed to the lowered pH (Girum et al., 2010). Moreover, *Entrococcus faecium* and *Lactobacillus fermentum* species isolated from fermented milk inhibit growth of *Helicobacter pylori*, *Escherichia coli* and *Salmonella typhimurium* (Thirabunyanon et al., 2008).

Traditional methods for preparation of fermented foods are not complicated and do not required expensive equipment. In the production of traditional fermented drinks (such as 'Ergo' and 'Tej') in Ethiopia, it is common to use and follow controlled natural fermentation process. Ergo is a traditional naturally fermented milk product, which has some resemblance to yogurt. The product is semisolid and has a pleasant odor and taste. Ergo fermentation is usually natural, with no defined starter cultures used to initiate it. This is made through the proliferation of the initial milk flora, with microbial succession determined by chemical changes in the fermenting milk (Esayas et al., 2008). Tej is also Ethiopian traditionally fermented beverage and produced from natural fermentation of water dissolved honey and Gesho (*Rhamnus prinoides*). It is alcohol product and can stay for a long period of time without spoilage.

Emerging and re-emerging of human pathogens due to food poisoning and food spoilage microorganisms is a current issue particularly in developing countries. However, there was no scientific report relating to the antimicrobial activity of LAB against food poisoning, food spoilage and pathogenic microorganisms, and how we fully exploited for use in Gondar. Thus, the present study was focused to isolate and determine antibacterial activity of LAB isolated from Ergo and Tej in this particular area.

MATERIALS AND METHODS

Study area and period

The study area was Gondar town. Gondar is one of the earliest cities in Ethiopia and located in North West Ethiopia which is 737 km away from Addis Ababa, capacity of Ethiopia (the only independent country in Africa and has its own letter and calendar) and African Union. Gondar is located in the North Gondar zone, Amhara region, North of Bahir Dar town and Lake Tana (the largest lake in Ethiopia) and South West of Simen Mountains. The town has 12°36′N latitude and 37°28′E longitude with an elevation of 2133 meters above sea level. According to 2008 Ethiopian statistical agency report, Gondar town has 231,977 total populations. The study was carried out from January 2013 to June 2013.

Sample collection and isolation of lactic acid bacteria

A total of 13 fermented beverage samples (8 for Ergo and 5 for Tej) were collected separately from Arada, Azezo and Kebele 18 using sterilized flasks and brought to the Microbiology laboratory, Department of Biology, University of Gondar. The samples were kept in the refrigerator (4 ^{0}C) until sample analysis was conducted within 24 hours.

One ml of each sample were taken and added separately in to test tube containing 9ml of normal saline (0.85% W/V) solution and mixed well using vortex mixer. Serial dilution were made up to 10^{-6} for each sample and 0.1 ml suspension from test tube containing 10^{-6} were taken and spread on MRS agar (selective medium for LAB) for each sample (Bali et al., 2011). The plates were incubated an anaerobically at room temperature for 3 days in an anaerobic jar and aerobically at 37 ^{0}C for 48 hrs in an incubator. The grown colonies having different morphology were subcultured using MRS slant and maintained at 4 ^{0}C for further analysis (Esayas et al., 2008).

Table 1. Lactic acid bacteria isolates isolated from Ergo and Tej in anaerobic and aerobic conditions

District	Sample type and their code		Isolates code	
			Anaerobic	Aerobic
Arada	**Ergo**	Ar01M	0	I_1, I_2
		Ar02M	0	0
		Ar03M	0	0
	Tej	Ar01T	0	0
		Ar02T	0	0
Azezo	**Ergo**	Az01M	0	0
		Az02M	0	0
	Tej	Az01T	0	I_3, I_4
Kebele 18	**Ergo**	K_{18}01M	0	0
		K_{18}02TM	0	0
		K_{18}03M	0	0
	Tej	K_{18}01T	I_5, I_6,…I_{16}	I_{17}, I_{18}
		K_{18}02T	0	0
Total			12	6

Key: I (designation of isolates)

Screening for antimicrobial activity of lactic acid bacteria isolates

The test organisms used for screening were standard (*Staphylococcus aureus ATCC2923* and *Escherichia coli ATCC2592*) and clinical (*Pseudomonas aeroginosa, Klebsiella pneumonia, S. aureus* and *E. coli*) human pathogens.

Lactic acid bacteria isolates were subcultured in sterile test tube containing MRS broth at 37 ^{0}C for 24 hrs and transferred in to sterile flask containing 150 ml MRS broth. And the broth culture was incubated at 37 ^{0}C for 3 days in thermostat water bath. To get the culture filtrate, 3days old culture was centrifuged at 10000 rpm for 20 minutes (Esayas et al., 2008).

Test (indicator) microorganisms were grown in a nutrient broth at 37 ^{0}C for 24 hrs. Wells were prepared on the surface of prepared nutrient agar plates using sterile cork borer (6mm in diameter). The entire surfaces of each nutrient agar plates were swabbed with test organisms by using sterile cotton swab. This procedure was repeated by streaking two more times, rotating the plate to ensure an even distribution of inoculums. Each well prepared on nutrient agar plates was filled with 50 µl culture free filtrates obtained from LAB isolates and Ampicillin. Antibacterial activity tests were done in triplicate and each plate was incubated at 37 ^{0}C for 24 hrs (Esayas et al., 2008). After incubation, the diameter of inhibition zone was recorded.

Determination of lactic acid and hydrogen peroxide production

Lactic acid bacteria isolates (I_2 and I_3) were grown on sterilized flask containing 300 ml MRS broth at 37 ^{0}C for 72 hrs in thermostat shaker water bath and the culture broth from each isolates was taken at 24 hrs interval to measure lactic acid and hydrogen peroxide production.

For 25 ml of broth culture of each isolates taken at 24, 48 and 72 hours incubation, 3 drops of phenolphthalein were added as indicator. A concentration of 0.1 N NaOH was prepared and placed in to the burette. From the burette 0.1 N NaOH was slowly added to the sample culture until pink color appears. Each ml of 0.1N NaOH is equivalent to 90.08 mg of lactic acid. Furthermore, to determine H_2O_2, 25 ml of dilute sulphuric acid was added to 25 ml of the broth culture of each isolates taken at 24, 48 and 72 hours incubation intervals. A concentration of 0.1 N potassium permanganate was prepared and the culture broth was titrated with 0.1N potassium permanganate up to decolourization occurs. Each ml of 0.1N potassium permanganate is equivalent to 1.070 mg of H_2O_2. A decolourization for each sample was regarded as end point (Saranya and Hemashenpagam, 2011).

Table 2. Comparison of mean inhibition zone of supernatants from isolates and standard Ampicillin (AMP) against test organisms

	Standard test organisms		Clinical test organisms			
	***E. coli* ATCC 2592**	***S. aureus* ATCC 2923**	***E. coli***	***S. aureus***	***K. pneumonia***	***P. aeroginesa***
I_2	16±1.7[b]	15.3±0.6[b]	14.7±1.5[b]	12.3±1.6[c]	14.3±0.6[c]	15±0[b]
I_3	14.3±0.6[b]	15.7±1.2[b]	14.3±1.2[b]	10±1[b]	13.3±0.6[b]	15±1[b]
AMP	11±1[a]	11.3±1.5[a]	6.7±0.6[a]	7±1[a]	10.3±0.6[a]	0±0[a]

Values are means ± SD. The outcomes not sharing a common superscript letter in the same column are significantly different at $p<0.05$.

Data analysis

Statistical Package for the Social Sciences (SPSS) 16 Version was used for analysis of recorded data using Microsoft word Excel 2007. The different inhibition zone measurements in triplicate were compared by performing One-way ANOVA ranked with Duncan's multiple range tests with descriptive analysis type. All statistical results with $p<0.05$ were considered to be statistically significant.

RESULTS

Sample collection and isolation of lactic acid bacteria

From a total of 13 samples collected, 18 isolates having different colony morphology were selected. From these isolates, 11% (2) from Arada, 11 % (2) from Azezo and 78% (14) from Kebele 18 isolates were found. Out of 18 isolates, 11% (2) isolates from Ergo and 89% (16) isolates from Tej were obtained. From these 18 isolates, 67% (12) and 33% (6) isolates were grown from anaerobic and aerobic conditions, respectively (Table1).

Screening for antibacterial activity of lactic acid bacteria isolates

From a total of 18 isolates, only 11 % (2) isolates were shown antibacterial activity against clinical and standard pathogens. There was high significance difference ($p<0.05$) among antibacterial activity of cell free supernatants from isolates (10±1 mm to 16±1.7 mm) and Ampicillin (0 to 11.3±1.5 mm) against clinical and standard human pathogens. The supernatant taken from isolate I_2 (16±01.7 mm) and I_3 (14.3±0.6 mm) was shown higher antagonistic activity against *E. coli* ATCC 2592 when compared to Ampicillin (11±1 mm). Moreover, antibacterial activity of filtrates from isolate I_2 (15.3±0.6 mm) and I_3 (15.7±1.2 mm) against *S. aureus* ATCC 2923 were shown higher when compared to Ampicillin (11.3±1.5 mm).

The supernatant from both I_2 (14.7±1.5 mm) and I_3 (14.3±1.2 mm) isolates were shown more antagonistic activity against *E. coli* (clinical isolate) when compared to Ampicillin (6.7±0.6 mm). Clinical isolate of *S. aureus* was more sensitive against supernatant from I_2 (12.3±1.6 mm) compared to I_3 (10±1 mm and) and Ampicillin (7±1 mm). Furthermore, *K. pneumonia* (clinical isolate) was more sensitive against supernatant from I_2 (14.3±0.6 mm) when compared to I_3 (13.3±0.6 mm) and Ampicillin (10.3±0.6 mm). Additionally, the antimicrobial activity of supernatant from both I_2 (15±0 mm) and I_3 (15±1 mm) were shown the most effective against *P. aeroginosa* (clinical isolate) compared to Ampicillin (0±0 mm) (Table2).

Determination of lactic acid and hydrogen peroxide

Much amount of lactic acid result were produced in 72 hrs incubation when compared to other incubation hours (24 and 48) for both I_2 and I_3 isolates. However, maximum amount of lactic acid was produced from isolate I_2 (1396.24 mg) when compared to isolate I_3 (810.72 mg) in this incubation period. As table 3 indicates that isolate I_2 was shown high amount lactic acid production not only at 72 hours incubation interval but also at 24 and 48 hours incubation when compared to isolate I_3. Moreover, the maximum amount of hydrogen peroxide (H_2O_2) also recorded at 72 hrs incubation time for both I_2 (3.7 mg) and I_3 (5.35 mg) compared to other incubation hours (24 and 48). As the result indicates, I_3 was shown much amount of H_2O_2 production when compared to I_2 in all incubation hr intervals (Table 3).

Table 3. Determination of lactic acid and hydrogen peroxide at 37^0C for different incubation hours

Isolates	Incubation hours	Measurements in mg	
		Lactic acid	Hydrogen peroxide (H_2O_2)
I_2	24	810.72	1.93
	48	873.78	2.14
	72	1396.24	3.7
I_3	24	720.64	3.10
	48	747.66	3.32
	72	810.72	5.35

DISCUSSION

Emerging and re-emerging of human pathogens are becoming the current issue worldwide. Thus, finding new antibacterial agents for the treatment of pathogens from microorganisms like lactic acid bacteria is a basic and important task. Lactic acid bacteria can produce different types of metabolites having antibacterial, antifungal, and anticancer and probiotic activities in different fermented drinks. Thus, potential LAB that produces antimicrobial agents can be isolated from different fermented beverages from different environmental conditions (Esayas et al., 2008). Moreover, the antimicrobial activity of metabolites produced from antibiotic producing microorganisms can be influenced by the type of the pathogen used, the physical and chemical parameters used under different environmental conditions (Abebe et al., 2013).

In the present study, from a total of 18 isolates, only 11% (2) of the isolates were shown antibacterial activity against clinical and standard pathogenic bacteria. The inhibition zone of cell free supernatant from these isolates against clinical and standard strains of human pathogens was ranged from 10±1 mm to15±1 mm and 13±1.7 mm to 16±1.7 mm, respectively. According to Savadogo et al (2004) reported that the inhibition zone of the supernatant from LAB isolates against standard human pathogens was ranged from 8 mm to 10 mm. Esayas et al (2008) also reported that the inhibition zone of supernatant of against human pathogens was ranged from 9.25 ± 0.35mm to 11.8 ± 0.35 mm. Thus, the present result was good when compared to Savadogo et al (2004) and Esayas et al (2008) results even if there are different determinant factors.

According to Saranya and Hemashenpagam (2011) report, the inhibition zone of cell free supernatant against clinical strains of *E. coli*, *K. pneumonia, P. aeroginosa* and *S. aureus* was ranged from 6 to14 mm, 7 to 14mm, 8 to 25mm and 6 to13 mm, respectively. The present study showed that the inhibition zone against clinical strains of *E. coli*, *K. pneumonia*, *P. aeroginosa* and *S. aureus* was ranged from 14.3±1.2 mm to 14.7±1.5 mm, 13.3±0.6 mm to 14.3±0.6 mm, 15mm to 15±1 mm and 10±1mm to12.3±1.6mm, respectively. As indicated here, except inhibition zone against *P. aeroginosa,* the present study result was shown good inhibition zone against *E .coli, K. pneumonia* and *S. aureus* when compared to Saranya and Hemashenpagam (2011) result.

During the present study, the amount of lactic acid and hydrogen peroxide production produced by selected isolates was ranged from 720.64 mg to1396.24 mg and 1.93 to 5.35 mg, respectively. Saranya and Hemashenpagam (2011) reported that the amount of lactic acid and hydrogen peroxide product produced by LAB isolates was ranged from 8.7 to 11.6 mg and 12 to 20 mg, respectively. Thus, our isolates have high potential for the production of Lactic acid and low potential for the production of hydrogen peroxide when compared to Saranya and Hemashenpagam (2011).

CONCLUSION

Lactic acid bacteria can produce different metabolites having antimicrobial and probiotic activities in different fermented drinks and foods. However, the production of these antibacterial agents can be influenced by biological (the type of starter organism used), physical and chemical parameters under different environmental conditions. Our result strongly support that the cell free supernatant of lactic acid bacteria isolated from fermented beverages (Ergo and Tej) have good antibacterial activity against clinical and standard human pathogens. Cultivation of these potential isolates under optimum condition can lead for the production of potential antibacterial agents (like lactic acid, hydrogen peroxide and others) having preservation as well as probiotic activity. These can reduce and control different human health problems caused by human pathogens. Therefore, isolation and screening of lactic acid bacteria from potential locally fermented drinks are the basic sources for the discovery of new potential LAB for

controlling and treatment of infectious disease to improve the health quality of human beings.

Conflict of interest statement

We declare that we have no conflict of interest.

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