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ADDIS ABABA UNIVERSITY,

FACULTY OF SCIENCE

SCHOOL OF GRADUATE STUDIES

BIOTECHNOLOGY PROGRAM

Antibiotic production and optimization of culture condition of Actinomycetes from some Soda Lakes of Ethiopia.

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A thesis submitted to the school of graduate studies

Addis Ababa University

In partial fulfillment of the requirement for the degree of Master of Science in Biotechnology

Addis Ababa

July, 2011

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ACKNOWLEDGEMENTS

I am thankful to almighty God for giving me grace and mercy upon my life, and for His divine enablement without Him nothing would have been possible.

My sincere gratitude goes to the School of Graduate Studies, Addis Ababa University for giving me the chance to enroll in the Biotechnology program and Ministry of Education of Ethiopia for financial assistance.

I am sincerely grateful to my advisor Dr. Amare Gessesse for giving me an opportunity to be involved in his project, for his invaluable advice, guidance, and support during my study, for his critical and insightful comments and for detailed correction of this thesis.

I am deeply indebted to Mesfin Tafesse for his support and encouragement during the very beginning of my study.

I would like to thank, Yemiserach Mulugeta for commenting and guiding during the lab session, Truneshi shiferaw, for her technical and laboratory assistance, I am also thankful for Beletu Mekonen, Zenebcehe Ayetenew and Tigist Mengesha for their assistance during laboratory work.

I also appreciate my friends Demesachew Guadie and Hanna Habtemariam, who has been with me in many ways, and all of my friends who worked with me in microbial biotechnology laboratory, thank you so much for your love and care.

Finally, to my darling Families, my father Ato Mehabie Mulualem, my mother, w/ro Yeshi Yirsaw, my brother Getachew Mehabie and my sister Tigist Mehabie for your love, care, prayers and support. In fact, for all. I say thank you.

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List of Abbreviations

ATCC	American Type Culture Collection
CF	Culture filtrate
^{o}C	Degree Celsius
EHNRI	Ethiopian Health and Nutrition Research Institute
LCHACT	Lake chitu Actinomycets
LSHACT	Lake shalla Actinomycetes
LABACT	Lake Abijata Actinomycets
MHA	Muller Hinton Agar
MIC	Minimum Inhibitory Concentration
MRSA	Methicilin Resistant *Staphylococcus aureus*
MDRTB	Multi Drug Resistance Tuberculosis
NA	Nutrient Agar
NB	Nutrient broth
OD	Optical Density
PDA	Potato Dextrose Agar
rpm	Revolution per minutes
SCA	Starch Casein Agar
SCB	Starch Casein broth
WHO	World Health Organization

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Abstract

The demand for new antibiotics increased due to the misuse and over use of antibiotics in various sectors creating an evolutionary pressure and resulting in multi drug resistant strains which result in increment of morbidity and mortality in the world. The main objective of this study was to explore the potential of alkaliphilic actinomycetes isolated from three Ethiopian soda lakes. About 171 isolates were screened for antibiotic production using cell free culture supernatant taken from the submerged fermentation and ethyl acetate crude extract of the culture, of which 93.6% of them showed antibiotic activity with cell free culture supernatant and 76 % of them with crude ethyl acetate extract against the test microorganisms used. Based on broad spectrum activity, zone of inhibition obtained and their activity with the use of crude ethyl acetate extract, two isolates LCHACT17and LABACT21 were chosen for further characterization. Antibiotic production was favored when phosphate concentrations was 0.2 % for LCHACT 17 and for LABACT 21. Yeast extract and sodium nitrate (for LCHACT 17) and combination of casein and potassium nitrate (for LABACT 21) were suitable nitrogen sources for antibiotic production. From the carbon sources used soluble starch was the best carbon source for antibiotic production by LCHACT17 while sucrose was found to be preferred carbon source by LABACT 21. Similarly it was observed that 1% sodium chloride concentration was favorable for antibiotic production by both isolates and the pH parameter indicated that pH10 (for LABACT 21) and pH11 (for LCHACT17) were the optimum pH for antibiotic production. The cell free culture supernatant of both isolates was stable at alkaline pH and decreases in activity at lower pH (pH 6 and below). The cell free culture supernatant of LCHACT17 was stable up to 60°c while LABACT 21 was stable up to 40 ^{o}c. The minimum inhibitory concentration (MIC) of LCHACT17 crude ethyl acetate extract against Streptococcus pneumonia was 12.5 mg/ml.

Key words/ phrases: Alkaliphiles, Antibiotics, Disk diffusion.

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1. Introduction

Since the discovery of penicillin in the late 1920s, hundreds of antimicrobial agents have been developed for human and animal use, which has been reduced the morbidity and mortality associated with numerous infectious diseases. This use has also resulted in an unprecedented global increase for the incidence of clinical bacterial strains that are multiple resistant to antibiotics (White and McDermott, 2001). Resistance has been a problem since man began to use antibiotics, and reflects the Darwinian selection where by these drugs kill susceptible bacteria but allow resistant ones to survive (Livermore and Dudley, 2000). The emergence of resistance is typically first observed in hospitals, where the lion's share of antimicrobials is used, then later among community bacterial isolates, presumably those transferred outside the hospital environment or selected by outpatient drug use (White and McDermott, 2001).

Bacteria resistant even for reserved antibiotics were reported, such as vancomycin-intermediate *Staphylococcus aureus* (Hiramatsu *et al*., 1997); on a wider scale there was a rising prevalence of methicillin-resistant *S. aureus*, vancomycin resistant *enterococci*, penicillin-resistant *pneumococci* and *Enterobacteriaceae* with potent -lactamases. Further problems, predominantly for developing countries, include multi-resistant *Mycobacterium tuberculosis* (Livermore and Dudley, 2000).

The demand for new antibiotics continues to grow due to the rapid spread of antibiotic-resistant pathogens causing life-threatening infections. Although considerable progress is being made within the fields of chemical synthesis and engineered biosynthesis of antimicrobial compounds, nature still remains the richest and the most versatile source for new antibiotics (Koehn *et al*., 2005). In the areas of cancer and infectious disease, 60% and 75% of new drugs, respectively, originated from natural sources between 1981 and 2002 (Newman *et al*., 2003). Between 2001 and 2005, 23 new drugs derived from natural products were introduced for the treatment of bacterial and fungal infections, cancer, diabetes, dyslipidemia, atopic dermatitis, Alzheimer's disease and genetic diseases such as tyrosinaemia and Gaucher disease (Lam, 2007).

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So far different antimicrobial agents have been obtained from natural sources. It has been reported that streptomycin, adriamycin, amphoteracin, bleomycin, cycloserine, erythromycin, gentamicin, kanamycin, lincomycin, neomycin, nystatin, oleandomycin, paramomycin, rifampicin, spectinomycin and viomycin was obtained from actinomycetes, largely from the *Streptomyces* genus. Bacitracin and Polymyxin were obtained from true bacteria, *Bacillus subtilis* and *Bacillus polymyxa* respectively. Cephalosporin C and cephalosporin G were produced by fungal species *Cephalosporium acremonium* (Amyes, 2001).

Filamentous bacteria belonging to the order *Actinomycetales*, especially *Micromomospora* and *Streptomyces* strains, have a unique and proven capacity to produce novel antibiotics (Watve *et al*., 2001). Many *Actinomycetes* are well known for their economic importance as producers of biologically active substances, such as antibiotics, vitamins and enzymes (McCarthy and Williams, 1992; Sanglier *et al*., 1996; Horan, 1999; Lazzarini *et al*., 2000). Similarly Hamaki *et al.,* 2005 suggested that, actinomycetes are important microorganisms since more than 90% of practical antibiotics originate from them and two thirds of 10,000 biologically active substances of microbial origin are produced from them (Berdy, 2005;Lazzarini *et al*., 2000). However, to discover commercially significant secondary metabolites from well known actinomycetes are becoming difficult as this practice leads to the wasteful rediscovery of known bioactive compounds. This, therefore necessitates the need to isolate, characterize and screen representatives of undiscovered actinomycete taxa. It is also becoming increasingly clear that habitats that were not explored before such as desert biomes and marine ecosystems, have a potential to be a rich sources of novel actinomycetes which have the capacity to produce interesting new bioactive compounds, including antibiotics (Bredholt *et al*., 2008).

Ulukanli and Digrak, (2002) suggested that screening bacteria from alkaline habitats or those grown under extreme cultural conditions remains a profitable area for investigation of new antibiotics. It has been reported that *Streptomyces sannanensis*, an alkaliphilic actinomycetes isolated from the alkaline soil of Saurashtra University Campus, Rajkot was capable of producing antibiotics that were active against Gram-positive bacteria (Vasavada *et al*., 2006). In another report an alkaliphilic actinomycete, *Streptomyces tanashiensis* strain A2D isolated from soil of phoomdi in Loktak Lake of Manipur, India exhibits a broad spectrum of antimicrobial

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activity against fungi and bacteria (Singh *et al*., 2009). In a similar fashion Biniam Wondale, (2008) reported that alkaliphilic actinomycetes, isolated from Ethiopian soda lakes were able to produce antimicrobial metabolites that were active against bacteria and fungi.

Hence the current study has been designed to isolate and screen alkaliphilic actinomycetes for new bioactive compounds from three soda lakes, Lake Chitu, Lake Shalla and Lake Abijiata in Ethiopia.

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2. Objectives

2.1. General objective

- § To isolate alkaliphilic actinomyecets and to explore their antibiotic production potential from soda lakes of Ethiopia, Lake chitu, Lake Shalla and Lake Abijiata.

2.2. Specific objectives

- § To isolate alkaliphilic actinomycetes using different selective cultivation media.
- § To test antibiotic production and efficiency of isolates using cell free culture filtrate and crude ethyl acetate extract on different test microorganisms.
- § To optimize the suitable cultivation media for production of antibiotics.

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3. Literature review

3.1. Antibiotics

Even if treatment of disease is almost as old as disease itself, the earliest efforts aimed at specific eradication of the disease causing agent from the patient is credited to Paul Erhlich. In the late 1800s, Erhlich launched a systematic investigation of chemical agents which displayed antimicrobial activity in the laboratory for their therapeutic efficacy in the infected patient (Amyes, 2001). Most important among the desired characteristics of these agents were: (*i*) the ability to quickly and specifically kill the infectious microbe; and (*ii*) to have no serious ill effects on the human host. For these reasons this agents were referred as "Magic Bullets". Since 1906, His effort led him to the discovery of salvarsan which became the first chemotherapeutic agent in clinical use to treat syphilis (Amyes, 2001). After that, the discovery of penicillin by Alexander Fleming in 1928 and its subsequent wide clinical application in 1940s; the discovery of Streptomycin, the first TB drug in 1943, by Selman Waksman and Albert Schatz have played remarkable role in the advancement of chemotherapy. Waksman was also the one who coined the term 'antibiotics'. Thus, antibiotics have been used to treat bacterial infections since the 1940s (Cloutier, 1995).

Antibiotics, in the broadest sense, are substances produced by living organisms, which, in small amounts, can inhibit the life processes of other organisms. The word antibiotic, in stricter sense, is used for a chemical substance, of microbial origin, that has the capacity to inhibit the growth of bacteria and other micro-organisms and even to destroy them (Burkholder, 1959). Antibiotics, literally "against life", are chemical compounds produced by actinomycetes, fungi, or bacteria that interfere with some essential bacterial structure or processes with no effects on the eukaryotic host having the infectious agents (Walsh, 2003). Although the term antibiotic was first introduced by Waksman in 1942 to denote chemical substances produced by a microorganism, semi synthetic modifications of natural products have produced a large variety of antibacterial agents, such as lactam antibiotics and macrolides that are also called antibiotics (Mascaretti, 2003). Currently there are three classes of chemically synthesized antibacterial agents in clinical use: the sulfa drugs introduced in the 1930s, the quinolones introduced in the 1960s, and oxazolidinone approved in the United States in 2000 (Lebb, 2004; Mascaretti, 2003).

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Antibiotics (including synthetic antimicrobials) are probably the most successful therapeutic agents developed by humans. Although sulphonamides, the first widely used antibiotics, are synthetic, the large majority of these compounds have a natural origin. Furthermore, production of antibiotics is a frequent characteristic of environmental microorganisms (Fajardo *et al*., 2009). Microbes can sense, adapt and respond to their environment quickly and can compete for defense and survival by the generation of unique secondary metabolites. These compounds are produced in response to stress and many have shown value in biotechnological or pharmaceutical applications (Zhang *et al*., 2005). Since several of the antibiotics used for treating infections are synthesized by soil micro-organisms, it has been assumed that the function of these compounds in nature should be to inhibit the growth of the microbial competitors of the antibiotic producers (Waksman and Woodruff, 1940). Inhibiting competing cells would leave more nutrients for the survival of the secondary metabolites producing strain (Thangapandian *et al*., 2007). Indeed many secondary metabolites show antibacterial or other inhibitory activities (anti-tumor, antifungal) or may function as herbicides (Sanglier *et al*., 1993; Ponmurugan and Poornima, 2006).

The antibiotic-producing species manage to avoid suicide by mechanisms that include (i) modification (and thus detoxification) of the antibiotic by enzymes formed by the antibiotic producing strain, (ii) alteration of the antibiotic target in the producing cell, and (iii) a decreased inward permeability to the antibiotic after it has been excreted(Martin and Demain, 1980).

All secondary metabolites including antibiotics are synthesized by pathways which are often connected and influenced by primary metabolism i.e. the metabolic pathways of primary metabolism often supply the precursors of secondary metabolism (Drew and Demain, 1977). Thus, frequently an intermediate metabolite from primary metabolism serves as precursor for the biosynthesis of the antibiotic. Therefore, the composition of the culture medium, closely connected with the metabolic capacities of the producing organism, greatly influences the biosynthesis of antibiotics. Changes in the nature and concentration of carbon and nitrogen sources, phosphorus concentration and trace elements have been reported to affect antibiotic biosynthesis in different organisms (Martin and Demain, 1980).

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Antibiotics can be divided into “Bacteriostatic” drugs, which merely inhibit the growth of bacteria (i.e. keeps them in stationary phase), and “Bacteriocidal” drugs, which actually kill the bacteria. However, the distinction is not absolute, and may be influenced by growth conditions, bacterial density, test duration, and extent of reduction in bacterial numbers (Pankey and Sabath, 2004).

3.2 Antibiotic Resistance

Since their introduction for human therapy in the 1940s, antibiotics have shown to be a remarkable success and constitute one of the most relevant medical inventions for reducing human morbidity and mortality. Unfortunately, the greatest threat to the use of antimicrobial agents for therapy of bacterial infections has been the development of antimicrobial resistance in pathogenic bacteria, reducing the possibilities for infections’ treatment and jeopardizing medical procedures, such as organ transplantations or implants of prostheses, where infective complications are common and antibiotic therapy is needed to prevent or treat those infections (WHO, 2000).

Antibiotic resistance has been shown to have occurred rarely in bacteria collected before the antibiotic era (Hughes and Datta, 1983). Shortly after the introduction of each new antimicrobial compound, emergence of antimicrobial resistance is observed (Levy, 1997). The development of bacterial resistance to antibiotics is caused by rapid evolution of the bacterial genome under selective antibiotic pressure and by the selective pressure of the environment. Resistance may develop to any antibiotic. A continuous selective pressure of routinely used antibiotics is an important precondition for the increase in multi resistant strains. Resistant mutants usually survive in an environment in which several antimicrobials are present (Kolar *et al.*, 2001). The magnitude of the problem is significantly increased by the possibility of bacteria to transfer resistance determinants horizontally and by the mounting increase in the use (over-use and misuse) of antibiotics, which has created an enormous selective pressure towards resistant bacteria (Levy, 1992).

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Antibiotic resistance can be divided into natural resistance and acquired resistance. Natural or intrinsic resistance is an inherent capacity of bacterial species related to its genetic background and often involves the presence of low affinity targets, low cell permeability or efflux mechanisms. For example *Pseudomonas aeruginosa* exhibits low level resistance to fluoroquinolones or amino glycosides due to intrinsically expressed efflux pumps, and or inactivation of - lactam antibiotics due to chromosomal - lactamase (Islam, 2008). Where as in acquired resistance bacterial species which was normally sensitive to a specific class of antibiotics become resistant as a result of mutations in chromosomal genes targeted by the antibiotic and/ or acquisition of resistance determinants borne on plasmids, bacteriophages, transposons, and other mobile genetic material (Islam, 2008; Levy and Marshall, 2004).

Commonly those pathogenic bacteria achieve active drug resistance through three major mechanisms: (i) efflux of the antibiotic from the cell via a collection of membrane-associated pumping proteins; (ii) modification of the antibiotic target (e.g., through mutation of key binding elements such as ribosomal RNA or even by reprogramming of biosynthetic pathways such as in resistance to the glycopeptide antibiotics); and (iii) via the synthesis of modifying enzymes that selectively target and destroy the activity of antibiotics (Wright, 2005).

3.3. Antibiotics from Natural Sources

Because of the steeply rising costs involved in identifying and developing novel synthetic antimicrobial agents, efforts are increasingly being made to find 'natural' bioactive compounds. These should in theory require less chemical engineering and therefore reduce the cost and time required for the early developmental stages. Moreover, it could be possible that antimicrobials developed from natural sources are less likely to have the undesirable side-effects associated with some synthetic compounds (Lampinen, 2005). Thus Natural products remains to be the most promising source of antibiotics (Bull and Stach, 2007).

There are approximately 32,500 natural products reported from microbial sources (Antibase data base) including about 1,000 derived from marine microbes (Singh and Pelaez, 2008).However Infectious diseases are leading health problems with high morbidity and mortality in the developing countries (Black *et al*., 1982). Driving this ‘natural’ approach further is the need to

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match the rate at which some bacterial species are developing antimicrobial resistance. Some species now have multi-resistant strains that are becoming a serious risk to health, such as MRSA and vancomycin resistant *Enterococci* (VRE) (Lampinen, 2005). This multidrug resistance is presently an urgent focus of research and new bioactive compounds are necessary to combat these multidrug resistance pathogens. It is indisputable that new drugs, notably antibiotics are urgently needed to halt and reverse the relentless spread of antibiotic resistant pathogens which cause life threatening infections and risk of undermining the viability of healthcare systems (Talbot *et al*., 2006).

Common sources of antibiotics are found among the actinomycetes, molds, and bacteria that live abundantly in soils, composts, and other places. Larger organisms, such as mushrooms and lichens, also produce antibiotic substances which are active against diverse living systems, ranging from bacteria to human cancer. In many flowering plants and in some coniferous trees antimicrobial compounds, of unusual nature or with chemical structures not unlike those of some of the mold antibiotics, are known to occur. A few kinds of algae elaborate antibacterial substances. Some kinds of corals produce compounds which strongly inhibit the acid-fast group of bacteria, to which the tuberculosis germ belongs (Burkholder, 1959).

3.3.1. Actinomyctes as sources of Antibiotics

The name 'Actinomycetes' was derived from Greek 'aktis' (a ray) and 'mykes' (fungus) and given to these organisms from initial observation of their morphology. They are diverse group of gram-positive bacteria that usually grow by filament formation and have high G+C (>55%) content in their DNA. Actinomycetes has been classified to the order Actinomycetales (Superkingdom: Bacteria, Phylum: Firmicutes, Class: Actinobacteria, Subclass: Actinobacteridae) (Okami and Hotta, 1988). They are free living, saprophytic bacteria widely distributed in soil, water and colonizing plants showing marked chemical and morphological diversity but form a distinct evolutionary line of organisms (Goodfellow and O'Donnell, 1989).

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The screening of actinomycetes for antibiotic production has increased markedly within the past decades. Many thousands of these organisms have been tested for this purpose. As the initial success of penicillin resulted in great emphasis being placed on the screening of saprophytic molds during the years 1940-1945, similarly interest in the actinomycetes was renewed and given added impetus with the discovery of streptomycin. This interest was further enhanced by the failure to find outstanding and medicinally attractive low-toxicity factors from the higher fungi or the lower bacteria. In contrast, concentration of attention on the actinomycetes in screening programs has provided lot of success to the medical stream as the discovery of Chloromycetin, Aureomycin and Terramycin has markedly increased the number of infectious diseases which may be successfully treated with antibiotics. Furthermore, the discovery of these agents has stimulated the hope that other antagonistic strains, yet undiscovered among the actinomycetes, may provide additional useful tools from the standpoint of medicine, control of certain plant pathogens, and the like (Benedict, 1953).

The most striking properties of the actinomycetes are the extent to which they produce antibiotics; most of the amino glycoside antibiotics (streptomycin, neomycin, kanamycin, gentamicin, tobramycin, and lividomycin) are produced by them (Benveniste and Daviest, 1973). In line with this, Berdy, (1989) and Mayfield *et al*., (1972) reported that Actinomycetes are responsible for the production of two-thirds of the microbial derived antibiotics known today. Around 23,000 bioactive secondary metabolites produced by microorganisms have been reported and over 10,000 of these compounds are produced by actinomycetes, representing 45% of all bioactive microbial metabolites discovered (Berdy, 2005). Among actinomycetes, around 7,600 compounds are produced by *Streptomyces* species. Many of these secondary metabolites are potent antibiotics, which has made streptomycetes the primary antibiotic producing organisms exploited by the pharmaceutical industry (Berdy, 2005). At least 70 of the approximately 100 marketed antibiotics used for the treatment of infections in humans are derived from substances produced by Streptomyces spp., for example *Streptomyces aureofaciens* is an important industrial microorganism as a producer of chlortetracycline and tetracycline (Yang and Ling, 1989).

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Watve *et al.*, (2001) estimated that from the first report of streptothricin in 1942 and streptomycin a year later, the order Actinomycetales had yielded ~3,000 known antibiotics (90% of those from *Streptomyces*, an Actinomycetales genus). On the basis of past experience, these authors proposed that if streptomycetes (exclusively) were screened as widely as they had been in 1995, 15–20 antibiotics would be discovered each year for the next 50 years. Over the subsequent five decades, these ~1,000 new molecules would yield 20–40 new antibiotics for human clinical use, assuming that the historical trend of one marketed antibiotic for 25–50 novel molecules remains the same. Apparently, this projected discovery rate was too low to be economically viable because several large pharmaceutical companies closed their antibiotic discovery programs shortly after its publication. However, since infectious diseases continue to be a major health problem (Kerr and Lacey 1995), screening efforts particularly from actinomycetes need to be continued so as to meet the demand of new antibiotics against the resistant pathogens (Watve *et al.*, 2001).

3.4. Alkaline environments

There are two kinds of naturally occurring stable alkaline environments in the world. Those high Ca^{2+} environments (groundwater bearing high CaOH) and low Ca^{2+} environments which includes the soda lakes and deserts which are dominated by sodium carbonate (Grant *et al.*, 1990).

3.4.1. Soda lakes

Soda lakes represent the most alkaline naturally occurring environments on earth, with pH values generally greater than 10, occasionally reaching pH 12 (Grant, 1992). The lakes are characterized by the presence of large amounts of Na_2CO_3 (usually as $Na_2CO_3.10H_2O$ or $Na_2CO_3.2H_2O$) and depletion of Mg^{2+} and Ca^{2+} because of the insolubility of those cations as carbonate minerals under alkaline conditions. Conditions suitable for the formation of soda lakes are found in low Ca^{2+}, high Na^{+} arid and semi arid tropical or semi-tropical zones where intense evaporative concentration rates exceed inflow rates such that salts accumulate. Alkalinity develops by a shift in the CO_2/ HCO_3/CO_3^{2-} in the absence of significant amounts of Ca^{2+} (which would precipitate CO_3^{2-} from solution as $CaCO_3$) such lakes are also somewhat saline due to the concomitant increase in Cl^- (Grant, 1992).

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The Great Rift Valley running through East Africa is an arid tropical zone where tectonic activity has created a series of shallow depressions. These are often closed basins with no significant outflow, where groundwater and seasonal streams flowing from the surrounding highlands form permanent standing bodies of water. Surface evaporation rates allow the dissolved minerals to concentrate into alkali basins with Na^+, CO_3^{2-}, and Cl^- as the major ions, creating pH values ranging from 8.5 in the most dilute lakes, to around 12 in the most concentrated. The Ethiopian soda lakes located in the rift valley area are Lake Aranguadi, Lake Kilotes, Lake Abijata, Lake Shala, Lake Chitu, Lake Hertale and Lake Metehara (Grant, 2006).

3.4.1.1 Alkaliphiles and their biotechnological applications

Alkaliphiles are microorganisms that grow optimally at pH values above 9.0, often with pH optima between 10.0 and 12, while showing little or no growth at near neutral pH values of 6.5 (Horikoshi, 1999).

Despite the profoundly alkaline and sometimes saline conditions, the dilute soda lakes are regarded as being amongst the world's most productive aquatic environments with productivity rates in excess of 10gC m^{-2} d^{-1} presumably because of relatively high ambient temperatures, high light intensities and unlimited access to CO_2 in these carbonate-rich waters (Grant *et al*., 1990). Primary productivity in most of these lakes is due to the presence of dense populations of alkaliphilic cyanobacteria, notably *Spirulina* spp. With some contribution from alkaliphilic anoxygenic phototrophic Bacteria of the genus *Ectothiorhodospira* (Grant and Tindall, 1986).

Soda lakes also contain dense populations of aerobic organotrophic and alkaliphilic bacteria supported by the primary productivity, some of these bacteria are believed to have biotechnological potential as sources of alkali-stable enzymes (Duckworth *et al*., 1996). Alkaliphiles isolated from Ethiopian soda lakes which had the potential for production of alkali stable enzymes were reported, such as alkaline protease from alkaliphilic bacteria (Amare Gessesse *et al*., 2003); alkali stable xylanases (Amare Gessesse, 1998). Studies have shown that various alkaliphiles are known to be sources of Antibiotics (Satyanarayana *et al* ., 2005). It has been indicated that microorganisms isolated from the alkaline saline Lake Acyg.l in Turkey were screened for their activity against other micro-organisms (Eltem and Ucar, 1998). The preliminary results indicated that alkaline-saline lake isolates exhibited antimicrobial activity

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against *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Mycobacterium smegmatis*, and *Candida albicans*. The preliminary results have encouraged further research work to identify the metabolites produced by alkaliphilic bacteria (Eltem and Ucar, 1998). It has been reported that some new antibiotics were produced by certain bacteria when an alkaline medium with high alkalinity (pH 9 to 10.5) was used (Sato *et al.,* 1983). The alkaliphilic actinomycete Nocardiopsis strain, a producer of phenazine, successfully grew at pH 10.0 in culture medium (Tsai *et al*., 1995). The discovery of these bioactive compounds provides evidence that organisms from such environments are also capable of producing antibiotic-type compounds. Alkaliphilic producers of novel bioactive agents still await exploitation (Ulukanli and Digrak, 2002).

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4. Materials and methods

4.1. Sample collection

Sediment samples were collected from lakes, Chitu, Shalla, and Abijiata, alkaline lakes in the Rift Valley region of Ethiopia. The samples were stored at 4^oC till they were used for further study.

4.2. Isolation and screening of alkaliphilic actinomycetes for antibiotic production

4.2.1. Serial dilution

Sediment samples from each lake were serially diluted, from 10^{-1} to 10^{-7} in 0.85% percent saline solutions. One gram of sediment from each lake was added in to 9 ml of sterile saline solution in a test tube. Then sequentially 1ml of the mixture was transferred in to the second test tube there by giving 100 fold diluted solution, in a similar fashion the dilution was done up to 10^{-7} times. Then 100 µl of each dilution were spread on a plate containing appropriate Starch Casein Agar or Humic Acid Agar media suitable for the growth of actinomycetes.

4.2.2. Media preparation for isolation of actinomycetes

Actinomycetes were isolated using Starch Casein Agar (SCA) or Humic Acid agar (Singh *et al.*, 2006). Starch Casein Agar was composed of (g/l) soluble starch, 10; Casein ,0.3; KNO_3, 2; NaCl, 2; K_2HPO_4, 2; $MgSO_4.7H_2O$, 0.05; $CaCO_3$,0.02; $FeSO_4.7H_2O$, 0.01; agar, 18; and Na_2CO_3, 10; and distilled water, 1,000 ml. Humic Acid agar was composed of (g/l) humic acid, 1; Na_2HPO_4,0.5 ; KCl ,1.71 ; $MgSO_4.7H_2O$, 0.05; $FeSO_4.7H_2O$, 0.01; $CaCO_3$, 0.02; yeast extract,1 and ; agar, 18. Na_2CO_3 was sterilized (at 121^oC, 1b pressure for 15 min) separately as a 25% solution and added to the rest of the medium after cooling. To inhibit Gram negative bacterial contamination 1 ml nalidixic acid was added to the medium from stock solution prepared by dissolving 0.01g of nalidixic acid in 10 ml of 100% methanol by swirling and mixing in 37^oC water bath (Zaitlin and Susan, 2006).

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4.2.3. Test microorganisms

The following test microorganisms were used for antimicrobial susceptibility test during the screening process. Gram positive bacteria: *Staphylococcus aureus (ATCC25923)*; *Streptococcus pneumonia (ATCC6303)*; Gram negative bacteria: *Escherichia coli (ATCC25922), Pseudomonas aeruginosa (ATCC27853), Shigella dysenterea* (clinical isolate)*, Salmonella typhii (ATCC6539)* and Fungus, *Aspergillus niger.* All the test microorganisms were obtained from Ethiopian Health and Nutrition Institute (EHNRI) with the exception of *Aspergillus niger,* pure isolate which was obtained from Addis Ababa University Microbiology Laboratory.

4.2.4. Media for test microorganisms

Nutrient agar and Nutrient broth

For the antimicrobial susceptibility test and screening, those test bacteria were seeded on nutrient agar media. Twenty eight grams of nutrient agar (NA) (based on manufacturer's recipe) was dissolved in 1 liter of distilled water and sterilized at 121°C, 1b pressure for 15 min. Then about 25 ml of sterile NA media was poured on sterile petridishes and allowed to cool overnight before use. Similarly 13 g Nutrient Broth was dissolved in 1 l distilled water and sterilized. About 5 ml of sterile nutrient broth was added on sterile screw capped test tubes then inoculated with test microorganisms and incubated overnight at 37°C. The broth culture was adjusted to 0.5 McFarland standards before it was used.

Muller Hinton agar

For antibacterial metabolite susceptibility test, thirty eight grams of Muller Hinton agar based on manufacturer's recipe was dissolved in 1l of distilled water and sterilized at 121°C, 1b pressure for 15 min. Twenty five ml was poured in to sterile petri dishes so as to grow the test microorganisms (bacteria).

Potato dextrose agar

Forty one grams of potato dextrose agar based on manufacturer's recipe was dissolved in 1l of distilled water and autoclaved at 121°c and 15 Psi, in order to grow the test fungus (*Aspergillus niger*).

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Preparation of 0.5 McFarland standards

About 0.05 ml of 1 % $BaCl_2$ and 9.95 ml of 1% H_2SO_4 were mixed to give 0.5 McFarland standards by which approximately $1.5x10^8$ bacterial cell suspensions are expected to be present per ml of culture (Raja *et al*., 2010).

4.2.5. Screening of actinomycetes for antimicrobial activity

Those isolates that were thought to have the actinomycete morphology under microscopic examination, their attachment to the substratum agar media and observation of colony morphology with naked eye were screened for their ability to produce bioactive compound. The screening process was done using the standard disc diffusion method (Bauer *et al*., 1966). Each isolate was cultured in a 250 ml flask containing 100ml SCB with pH adjusted around 10-10.5 using Na_2CO_3 (Hozzein *et al*., 2004). Then submerged fermentation was done at room temperature in rotary shaker at 120 rpm for 14 days. Finally the 14th day of post inoculation broth culture was centrifuged at 5,000 rpm for 10 min (Hassan *et al*., 2001). The supernatant (culture filtrate) was tested for antimicrobial activity. The antimicrobial screening was done by impregnating about 100 µl of the supernatant on the disc paper which was manually prepared from 3 mm thick filter paper and then those disc papers were transferred with alcohol flame sterilized forceps in to nutrient agar (for bacteria) and potato dextrose agar (for fungus) previously seeded with the target test microorganisms.

4.2.6. Extraction of the crude antibiotic using ethyl acetate

Those isolates that showed antimicrobial activity while their culture supernatant was used were further studied by extracting their antibacterial metabolite with ethyl actetate 1/1(v/v) (Westley *et al*., 1979). In the process the organic phase which contained the crude antibiotic dissolved in ethyl acetate was separated from the remaining solutions in a separatory funnel. The ethyl acetate phase was allowed to evaporate in Rota vapor by which the ethyl acetate was recovered and the crude semi solid antibiotic was obtained. These crude extracts were used for the antimicrobial susceptibility test using the disc diffusion assay method.

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4.2.7. Selection of potent antibiotic producers

A total of 235 isolates (designated by an abbreviation, LCHACT for Chitu isolates, LSHACT for Shalla isolates and LABACT for Abijiata isolates) were isolated from three alkaline lakes. Out of these, 171 alkaliphillic microorganisms were screened for antimicrobial production. The best antibiotic producers were selected based on their broad spectrum activity, zone of inhibition and better antimicrobial activity with crude ethyl acetate extract for further characterization. Two isolates designated as, LCHACT17 (isolate number 17 from Lake Chitu) and LABACT21 (isolate number 21 from Lake Abijiata) respectively were selected for further characterizations. These isolates have showed relatively broader spectrum activity, better zone of inhibition and antimicrobial activity using the crude ethyl acetate extract on different test microorganisms.

4.3. Morphological and biochemical characterization of selected isolates

For identification of the selected isolates gram staining was done using KOH method as described in (Halebian *et al*., 1981). By which two drops of 3% solution of potassium hydroxide were mixed with a loopful of the 48hr culture on the slide. The loop was occasionally raised 1 to 2 cm from the surface of the slide. A string of the mixture would follow the loop when it was raised for gram negative bacteria, while absence of stringing indicates the bacteria is gram positive.

For Catalase test, isolates were placed on a clean glass slide. Then two drops of a 3% H_2O_2 solution were added to the glass slide and mixed with a clean tooth pick. Rapid formation of bubbles indicates that the isolates are catalase positive, while absence of bubble formation is an indication of being catalase negative (Collins *et al*., 1976).

Nitrate Reductase test, those selected isolates were grown in a nitrate containing broth (Starch Casein Broth), confined in a test tube for 48 hour at 30°c. Then a few drops of sulfanilic acid and N, N-Dimethyl- -naphthylamine were added to the test tubes containing the culture. The tubes were Shaken well to mix reagents with medium. A distinct red or pink color, which should develop within a few minutes, indicated nitrate reduction (positive result). While for those remained colorless, Zn powder was added and waited for 10-15 min so as to check whether the

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color change occurred or not. Being colorless after addition of Zn powder is a positive result while changing of the suspension in to pink/ red is a negative result (Collins *et al.,* 1976).

To determine for the presence of cytochrome oxidase system in the selected isolates, small piece of what man filter paper was soaked with 1%(w/v) N,N-dimethyl-P-phenyl diammonium chloride, then a Loopful of 24 hr old culture were scrapped and rubbed on the filter papers. Appearance of blue color within 10 to 30 seconds indicated a positive test for oxidase (Collins *et al*., 1976). And finally Starch hydrolysis test of the selected isolates was done using starch casein agar media. The appearance of clear zone after flooding of lugol's iodine around the growth area indicates positive result (Grimont *et al.*, 1977).

4.4. Optimization of culture condition for antimicrobial production by the selected isolates

4.4.1. Effect of different incubation periods on growth and production of antibiotic by the selected isolates

Growth and antibiotic production of selected isolates was studied over a period of 15 days and 18 days respectively on SCB media (Saha *et al*., 2010; Hassan *et al*., 2001). The growth was determined by measuring the turbidity of the cells using spectrophotometer at 600nm at different sampling days of incubation, and antibiotic production was examined from the 3rdday-18th day of post inoculation period using disc diffusion method.

4.4.2 Effect of different carbon source on production of antibiotic by selected isolates

To determine the effect of carbon sources on antibacterial metabolite production, different carbon sources such as Starch, Glycerol, Sucrose, Maltose, Lactose, D-glucose, D- fructose, D-Xylose, L-Rhamnose, L-Raffinose, and D-Mannitol were used. One percent of those carbon sources was separately autoclaved on a test tube and mixed to the basal media (without starch). The basal media without carbon source was used as a negative control and SCA was used as a positive control (Saha *et al*., 2010; Hassan *et al*., 2001).

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4.4.3. Effect of different nitrogen source on antibiotic production of by selected Isolates

To determine the effect of nitrogen source on antibacterial metabolite production different nitrogen sources such as Casein, Yeast extract, Peptone and Tryptone (from organic sources), Sodium nitrate ($NaNO_3$), Potassium nitrate (KNO_3), Ammonium nitrate (NH_4NO_3) and Ammonium sulfate ($(NH_4)_2SO_4$) (from inorganic sources) were used. Each nitrogen sources was added on 100 ml SCB at 0.25% concentration. SCA without nitrogen source was used as a negative control while SCA was used as a positive control (Saha *et al*., 2010; Hassan *et al*., 2001).

4.4.4. Effect of different concentration of phosphate on growth and antibiotic production by selected isolates

Different concentrations of dibasic potassium phosphate (K_2HPO_4) were used so as to determine the appropriate concentration for antimicrobial metabolite production. The different concentrations of K_2HPO_4 used were: 0.1 %, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 1%, 3% and 5%, basal media without K_2HPO_4 was used as a negative control while SCA was used as a positive control (Hassan *et al*., 2001).

4.4.5. Effect of different sodium chloride (salt) concentration on growth and antibiotic production by selected isolates

The effect of different concentration of sodium chloride on production of antimicrobial metabolite was determined by using these concentrations of NaCl: 0.1%, 0.2%, 0.3%, 0.5%, 0.7%, 1%, 2%, 5%, 7% and 9%. Starch casein broth without NaCl, was used as negative control while SCA was used as a positive control (Saha *et al*., 2010; Hassan *et al*., 2001).

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4.4.6 Effect of different pH on growth and antibiotic production by selected isolates

The effect of pH on the production of antimicrobial metabolite by selected isolates were evaluated by adjusting 100ml SCB in 250ml flask from pH 6 to pH 13 using 1N HCl and 25% Na_2CO_3 (additional 1N NaOH for pH above 11) (Saha *et al.*, 2010).

4.4.7. Determination of Final dry Weight and Final pH of the culture

The final dry weight (on incubation days by which the maximum antibacterial metabolite production detected) of the selected isolates cultured on different carbon sources, different nitrogen sources, different phosphate and different sodium chloride concentration was determined. In the process 10ml of the culture of selected isolates was taken and centrifuged at 5000 rpm for 10 min and the pellet was allowed to be dried in an oven at 70°C using evaporating dishes until a constant weight was gained. The final pH of the culture of those selected alkaliphilies grown on different carbon sources, nitrogen sources, different salt concentrations and different phosphate concentrations was measured in order to know whether the culture condition has remained alkaline or not (Hassan *et al.*, 2001).

4.4.8. pH and temperature stability of culture supernatants of selected isolates

The pH of culture filtrate of selected isolates was adjusted to pH 2, 4, 6 8, 10 and 12. Then its antibacterial activity was checked on different test microorganisms. The untreated culture filtrate was used as a positive control. Likewise the temperature stability of the culture filtrates was examined by adjusting to different temperatures 40°C, 50°C, 60°C, 70°C, 80°C, 90°C, 100°C and 121°C for about 30 min (Muiru *et al.*, 2007).

4.5. Determination of minimum inhibitory concentration of crude ethyl acetate extract

The minimum inhibitory concentration was determined using broth tube dilution method (Augustine *et al.*, 2005). About 100mg of crude ethyl acetate extract was dissolved in 1ml of 0.2M phosphate buffer (2ml of stock was prepared by mixing 200mg of crude extract with 2m of 0.2M phosphate buffer). Sterile capped test tubes containing 1ml of sterile nutrient broth was numbered from 1-9. Then 1m of the stock solution was added to test tube 1 containing 1m of

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sterile broth and mixed well with a pipette. Then 1ml from test tube 1 was taken and added to test tube 2 and mixed well with a separate pipette tip. Similarly the two fold dilution was done up to the 8th tube and finally 1ml from tube 8 was discarded and the 9th tube without extract served as a negative control as it didn't receive any antibiotic. All of the 9 tubes were inoculated with appropriate inoculums by diluting 0.2 ml of 0.5 Macfarland standardized inoculums in 40 ml of sterile nutrient broth. Then 1ml of suspension was added to all test tubes and incubated at 37°c for overnight. For each of the dilutions made a control was prepared with similar concentration of crude ethyl acetate extract and nutrient broth without cell, while only sterile nutrient broth serve as a control for test tube 9(negative control). After overnight incubation turbidity was measured using spectrophotometer at 600 nm.

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5. RESULTS

5.1. Isolation and Screening of alkaliphilic actinomycetes for antibiotic production

Out of 171 isolates screened for antibiotic production using submerged fermentation, 160 (93.6%) of them showed antibiotic activity with the cell free culture supernatant. And about 76% of them showed activity with the crude ethyl acetate extract. (Appendix 1, Figure 1).

5.2. Morphological and Biochemical characterization

In terms of color of colony both isolates were found to be white. But in terms of shape of colony LCHACT 17 was rod while LABACT 21 was filamentous. In terms of pigment production LCHACT 17 produce brown color while no color was produced by LABACT 21. On the other hand on all other parameters both isolates are similar i.e. both isolates are gram positive, catalase and oxidase reactions are positive, they are able to reduce nitrates to nitrites and able to degrade starch. (Table. 1.)

Table. 1: Morphological and biochemical characteristics

Type	LCHACT 17	LABACT 21
Color of colony	White	White
Shape of colony	Rod	Filamentous
Pigment production	Brown	No color
Gram reaction	+	+
Catalase test	+	+
Oxidase test	+	+
Nitrate reduction test	+	+
Starch hydrolysis	+	+

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5.3. Optimization of selected isolates for antibiotic production

5.3.1. Effect of different incubation periods on growth and production of antibiotic by LCHACT 17 and LABACT 21

As shown in Figure 1, isolate LCHACT 17 began to grow after 48h of post inoculation and showed maximum growth at the 7th day and gradually declining up to 15th day of post inoculation**.** While isolate LABACT 21 began to grow after 48hr of post inoculation and showed maximum growth at 6th day and gradually declining on the subsequent days of incubation.

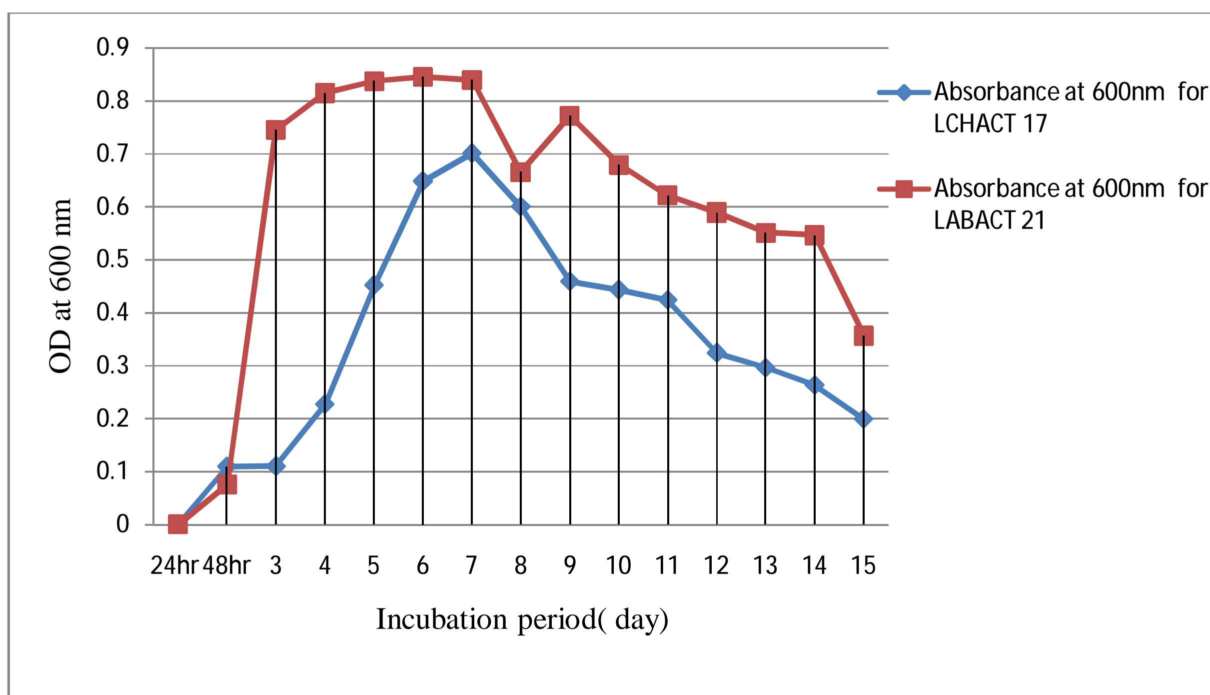

Figure 1: Effect of incubation period on growth of LCHACT 17 and growth of LABACT 21.

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The effect of the antimicrobial activity of culture filtrate of LCHACT17 on the different test microorganisms showed variation as shown in Table 2. Production of antibacterial metabolite was not observed up to 4 days of incubation. However, within 5-6 days of incubation antibacterial metabolite production was detected with maximum zone of inhibition of 8mm against *E. coli* and *S. aureus.* At the 7 day of incubation antibacterial activity was detected on all test microorganisms with the exception of *S. dysentrea.* Starting from 8 days to 12 days of incubation all test microorganisms were sensitive to the culture filtrate, with maximum activity detected at 12 days of incubation. Showing some decrement in activity starting from 13 days of incubation with least activity detected from 16 days and above.

Table. 2: Diameter of zone of inhibition (mm) of antibacterial metabolite of LCHACT 17 taken at different incubation period (day).

Date of incubation	*E. coli*	*S. aureus*	*P.aeruginosa*	*S. dysentrea*	*S. typhii*	*S. Pneumonia*
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	8	8	0	0	0	0
6	8	8	0	0	0	0
7	8	8	7	0	7	7
8	8	8	7	7	7	8
9	8	8	8	8	7	8
10	10	8	9	8	8	11
11	10	8	9	8	8	12
12	12	8	9	8	8	12
13	9	8	8	0	8	9
14	9	7	9	0	7	9
15	9	0	9	0	8	9
16	8	0	8	0	8	8
17	8	0	8	0	8	8
18	8	0	8	0	8	8

N.B. measurement includes the diameter of the disc (6mm).

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On the other hand for LABACT 21antibacterial metabolite production was not detected up to 8 days of incubation. Starting from the 9th day of incubation antimicrobial activity of the culture filtrate of LABACT 21 was detected on all test microorganisms with the exception of *E. coli*. Maximum activity was detected at 14th day, while decrement in activity was observed from 16 days onwards. (Table.3.)

Table. 3: Diameter of zone of inhibition (mm) of antibacterial metabolite of LABACT 21 taken at different incubation period (day).

Date of incubation	*E. coli*	*S. aureus*	*P.aeruginosa*	*S. dysentrea*	*S. typhii*	*S. Pneumonia*
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	8	8	8	8	8
10	0	8	8	8	8	8
11	0	8	8	9	8	8
12	0	8	8	9	8	8
13	0	9	9	9	8	8
14	0	9	11	12	9	9
15	0	9	10	12	9	9
16	0	8	9	11	8	8
17	0	8	9	11	8	8
18	0	8	9	10	8	8

N.B. measurement includes the diameter of the disc (6mm).

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5.3.2 Effect of different phosphate concentration on growth and antibiotic production by LCHACT 17 and LABACT 21.

From dry weight measurement (mg/ml) of LCHACT 17 and LABACT 21 it was observed that both isolates showed growth in all phosphate concentrations. But as phosphate concentration increased the biomass also increased, with minor deviation observed for LABACT 21. (Figures. 2 and 3).

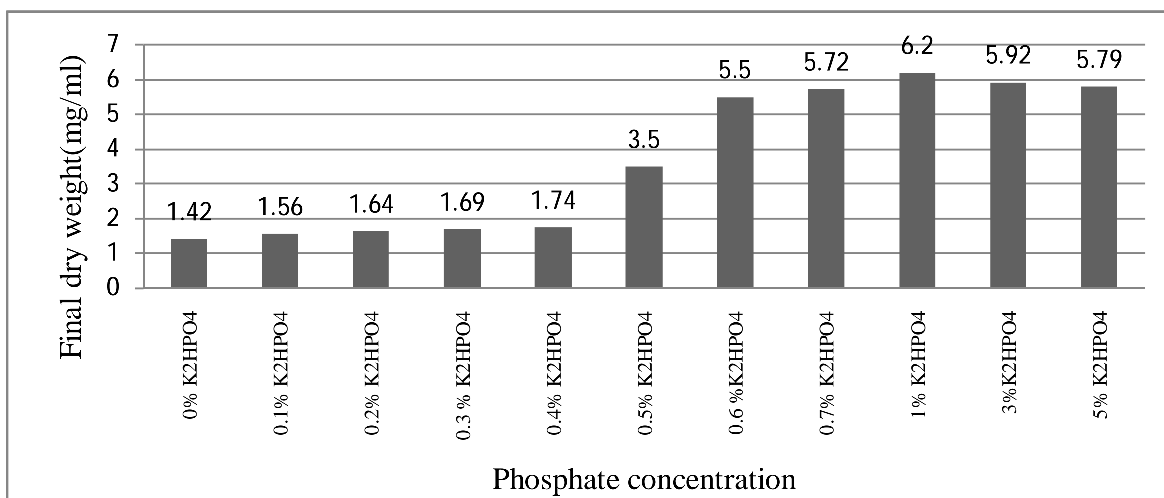

Figure.2. Drybiomass of LCHACT 17, at 12th day on different phosphate concentration**.**

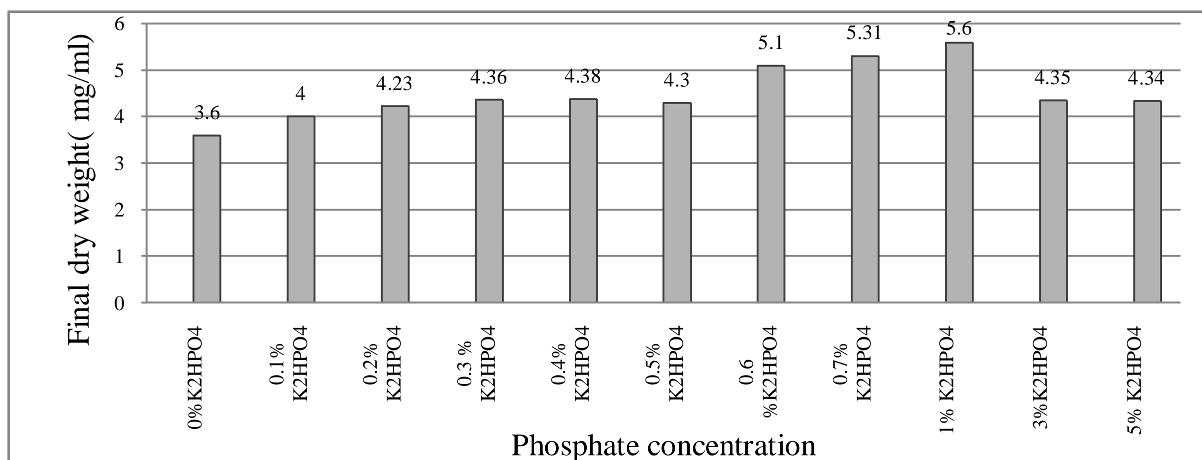

Figure. 3: Dry biomass of LABACT 21, at 14th day on different phosphate concentration.

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When LCHACT 17 was grown at different phosphate concentration and antibacterial metabolite production tested from 12 day culture filtrate, detectable activity was observed at the concentration range of 0.1-0.7% (Appendix 2, Figure 2A) with maximum activity detected at 0.2%. No antibacterial metabolite production was detected when the phosphate concentration was increased to 1% and above (Table. 4).

Table.4: Antibacterial activity of LCHACT 17 (12th day extracted CF on different K_2HPO_4 concentration).

Phosphate (K2HPO4) Concentration	Test microorganisms and zone of inhibition in mm						
	E.coli	*S.aureus*	*P.aeruginosa*	*S.dysentrea*	*S.typhii*	*S.pneumonia*	Final pH
0%	0	0	0	0	0	0	9.72
0.1%	0	8	8	0	8	8	9.68
0.2%	9	9	10	0	8	9	9.63
0.3%	8	8	8	0	8	8	9.61
0.4%	8	8	8	0	8	8	9.59
0.5%	8	8	9	0	8	9	9.60
0.6%	8	8	8	0	9	8	9.58
0.7%	8	8	8	0	8	12	9.53
1%	0	0	0	0	0	0	9.54
3%	0	0	0	0	0	0	9.51
5%	0	0	0	0	0	0	9.55

N.B. measurement includes the diameter of the disc (6mm).

However, when the growth period was extended to 21 days, antibacterial metabolite production was observed even at higher phosphate concentrations (Table. 5).

Table .5: Diameter of zone of inhibition LCHACT 17 grown on 1%, 3%and 5% phosphate concentration (CF extracted on 21th incubation day).

Phosphate concentration	Test microorganisms and diameter of zone of inhibition					
	E.coli	*S.aureus*	*P.aeruginosa*	*S.dysentrea*	*S.typhii*	*S.pneumonia*
1%	8	9	9	0	9	8
3%	8	9	9	0	8	0
5%	8	9	8	0	8	8

N.B. measurement includes the diameter of the disc (6mm).

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When LABACT 21 was grown on different phosphate concentration and antibacterial metabolite production tested from the 14 day culture filtrate, maximum activity was detected at 0.2% (Appendix 2, Figure 2B) and a decrease in activity was observed in a range of 0.3%-0.7% concentration (Table.6.). On the other hand no antibiotic was produced at 1% and above phosphate concentration.

Table. 6: Diameter of zone of inhibition of LABACT 21 on different Phosphate concentration (CF extracted on14th day).

Phosphate (K_2HPO_4) Concentration	Test microorganisms and zone of inhibition in mm						
	E. coli	*S.aureus*	*P.aeruginosa*	*S.dysentrea*	*S.typhii*	*S.pneumonia*	Final PH
No phosphate	0	0	0	0	0	0	9.88
0.1%	0	9	9	8	8	0	9.55
0.2%	10	12	9	8	9	9	9.80
0.3%	0	8	9	8	8	0	9.76
0.4%	0	8	8	9	8	0	9.90
0.5%	0	8	8	8	8	0	9.87
0.6%	0	8	8	8	8	0	9.58
0.7%	0	8	8	8	8	0	9.60
1%	0	0	0	0	0	0	9.39
3%	0	0	0	0	0	0	9.56
5%	0	0	0	0	0	0	9.42

N.B. measurement includes the diameter of the disc (6mm).

However when the day of incubation was extended to 21 day, culture filtrate of LABACT 21 grown on 1%, and above phosphate concentration showed good antibacterial activity (Table. 7).

Table.7: Diameter of zone of inhibition of LABAC21 on different phosphate concentration (CF extracted on 21th day).

Phosphate concentration	Diameter of zone of inhibition(mm) and test microorganisms					
	E.coli	*S.aureus*	*P.aeruginosa*	*S.dysentrea*	*S.typhii*	*S.pneumonia*
1%	0	9	8	8	0	8
3%	0	9	8	8	0	8
5%	0	9	8	8	0	8

N.B. measurement includes the diameter of the disc (6mm).

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5.3.3. Effect of different carbon sources on growth and antibiotic production by LCHACT 17 and LABACT 21

From dry weight measurement (mg/ml) of LCHACT17 maximum growth was detected in the presence of starch followed by D- xylose, D-glucose and Glycerol respectively, least growth was observed with lactose, L-rhamnose and L-raffinose respectively. Whereas for LABACT21 the maximum dry weigh was obtained when D-xylose followed by starch and glycerol respectively were used as carbon sources in the cultivation media. Least was observed when lactose used as carbon source. (Figures. 4 and 5).

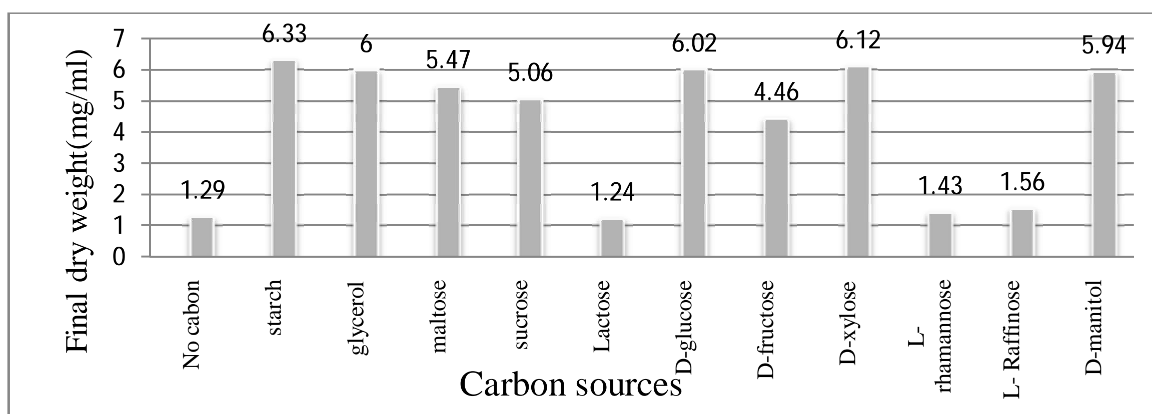

Figure.4: Final dry weight of LCHACT 17 grown on different carbon sources

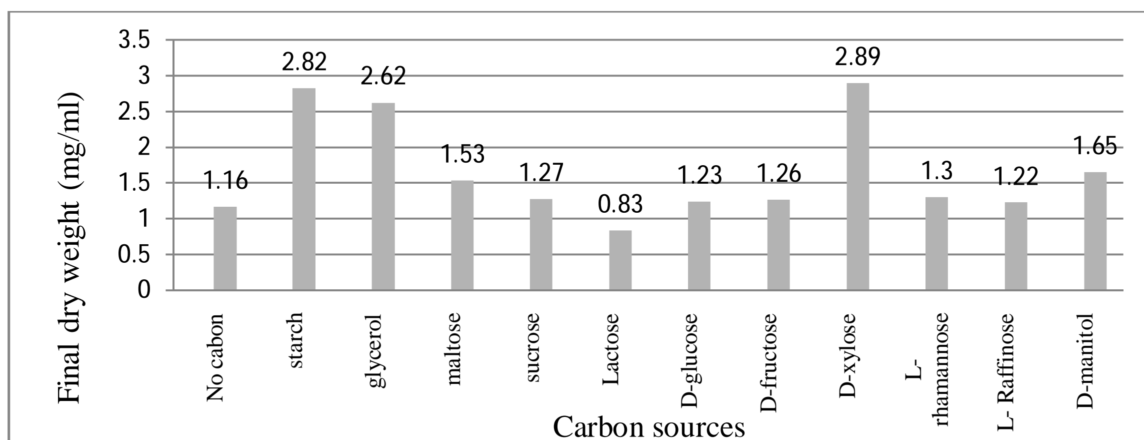

Figure. 5: Final dry weight LABACT 21 grown on different carbon sources

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As shown in Table 8 below for LCHACT17 high antibacterial metabolite production was obtained by the presence of starch (Appendix 2, Figure.3A), followed by glycerol, D- xylose, maltose and D-glucose as the only carbon sources in the submerged fermentation. In terms of effectiveness of antibacterial activity, the antibacterial metabolite produced from different carbon sources was more effective on *E.coli, S.typhii, P.aeruginosa, S.pneumonia, S.aureus* respectively and no activity was detected against *S.dysentrea.*

Table. 8: Diameter of zone of inhibition of LCHACT 17 grown on different carbon sources

Carbon sources	Test microorganisms and zone of inhibition in mm					
	E.coli	*S.aureus*	*P.aeruginosa*	*S.dysentrea*	*S.typhii*	*S.pneumonia*
No carbon	0	0	0	0	0	0
Starch	11	10	9	0	12	9
Glycerol	10	10	9	0	9	11
Sucrose	8	0	9	0	8	8
Maltose	9	9	9	0	9	9
Lactose	0	0	0	0	8	0
D-glucose	8	8	9	0	8	8
D-fructose	8	0	9	0	8	0
D-Xylose	10	10	9	0	8	9
L-Rhamnose	0	0	0	0	8	0
L-Raffinose	8	8	0	0	0	0
D-Manittol	8	0	8	0	0	8

N.B. measurement includes the diameter of the disc (6mm).

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As shown in Table 9 below for LABACT21 high antibacterial metabolite production was detected by the presence of sucrose (Appendix 2, Figure 3B), followed by maltose, glycerol, D-xylose respectively. In terms of susceptibility *S.dysentrea, E.coli* and *S.aureus* respectively were more sensitive to antibacterial metabolite of LABACT21 produced from different carbon sources. *S.typhii* , *P.aeruginosa* and *S.pneumonia* respectively were less sensitive.

Table. 9: Diameter of zone of inhibition of LABACT 21 grown on different carbon sources

Carbon sources	Test microorganisms and zone of inhibition in mm						
	E.coli	*S.aureus*	*P.aeruginosa*	*S.dysentrea*	*S.typhii*	*S.pneumonia*	Final pH
No carbon	0	0	0	8	0	0	10.02
Starch	8	8	0	8	0	0	9.88
Glycerol	8	8	0	11	0	0	10.03
Sucrose	10	8	8	9	8	8	9.84
Maltose	8	8	0	8	0	8	9.94
Lactose	0	0	0	0	0	0	9.87
D-glucose	8	0	0	9	0	0	10.01
D-fructose	8	0	0	8	0	0	9.91
D-Xylose	9	8	0	8	0	0	9.93
L-Rhamnose	0	0	0	0	0	0	9.90
L-Raffinose	8	8	0	8	0	0	9.93
D-Manittol	8	8	0	8	0	0	9.90

N.B. measurement includes the diameter of the disc (6mm).

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5.3.4. Effect of nitrogen source on growth and antibiotic production by LCHACT 17 and LABACT 21

The maximum final dry weight (mg/ml) for LCHACT17 was obtained in cultivation media containing tryptone as the only nitrogen source and the minimum was obtained by $(NH_4)_2SO_4$. Whereas for LABACT 21 the maximum final dry weight was obtained in cultivation media containing peptone as the only nitrogen source and the minimum was obtained by KNO_3. (Figures. 6 and 7).

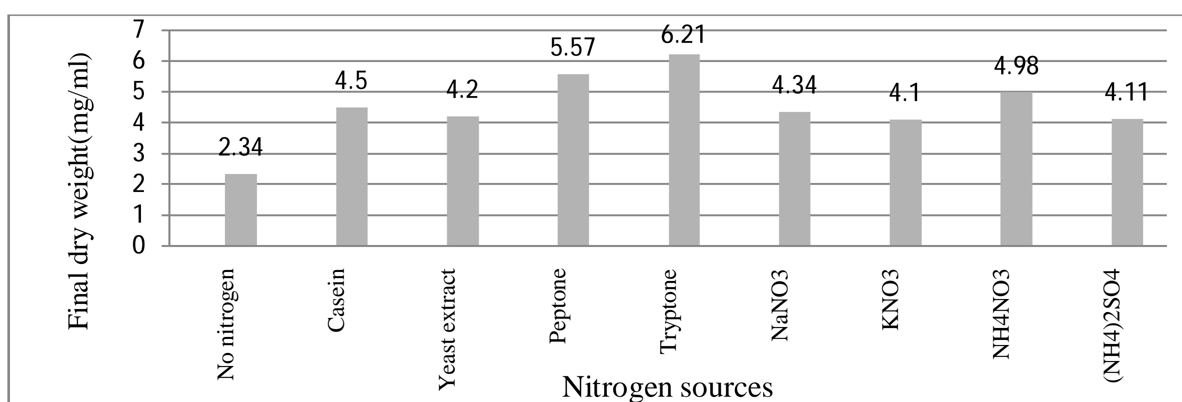

Figure.6: Final dry biomass of LCHACT 17 on different nitrogen source (12th day).

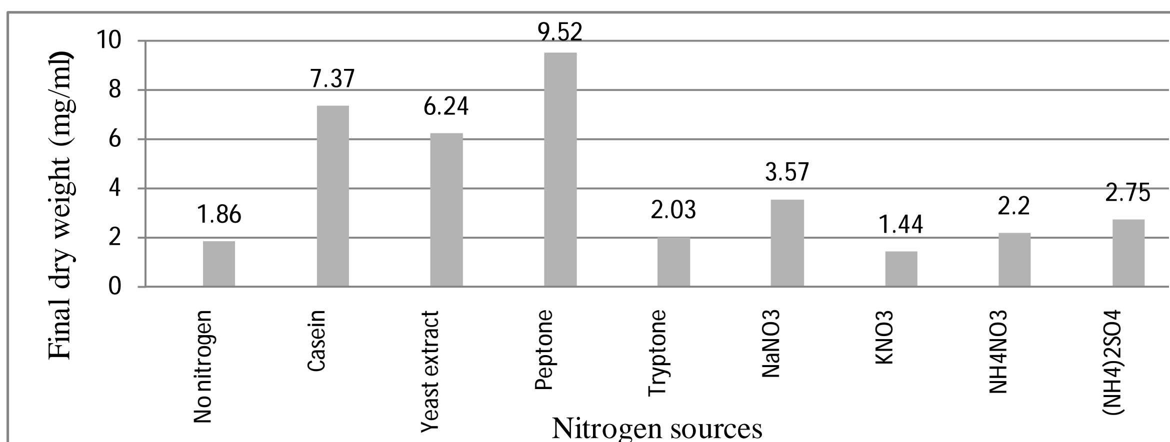

Figure.7 : Final dry weight of LABACT 21 grown on different nitrogen sources

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As shown in Table 10 below production of antibacterial metabolite by LCHACT 17 has given maximum zone of inhibition in the presence of yeast extract against *E.coli* and *S.aureus* (Appendix 2, Figure 4A). Whereas in terms of multiple inhibition of the test microorganisms and inhibition zone $NaNO_3$ (Appendix 2, Figure 4B), Casein and KNO_3 were suitable nitrogen sources respectively, while yeast extract and $(NH_4)_2$ SO_4 were least effective in multiple inhibition of the test micorganisms. In terms of sensitivity to antibacterial metabolite of LCHACT17 extracted from different nitrogen sources, *E.coli* and *S.aureus* were more followed by *P.aeruginosa*, *S.dysentrea* and *S.typhii* while *S.pneumonia* was not susceptible.

Table.10: Diameter of zone of inhibition in mm of LCHACT 17 grown on different nitrogen sources.

Nitrogen sources	Test microorganisms and zone of inhibition in mm						
	E.coli	*S.aureus*	*P.aeruginosa*	*S.dysentrea*	*S.typhii*	*S.pneumonia*	Final pH
NoNitrogen source	0	0	0	0	0	0	9.95
Casein	12	12	9	11	9	0	9.47
Yeast extract	14	17	0	0	0	0	9.74
Peptone	9	9	9	9	8	0	9.44
Tryptone	9	9	9	9	9	0	9.63
$NaNO_3$	12	12	10	11	12	0	9.71
KNO_3	12	11	9	9	10	0	9.72
NH_4NO_3	10	9	8	8	8	0	9.40
$(NH_4)_2$ SO_4	8	9	8	8	0	0	9.15

N.B. measurement includes the diameter of the disc (6mm).

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As shown in Table 11 below antibacterial activity of CF of LABACT 21 grown in individual nitrogen sources was very weak as compared to the antibacterial activity obtained from the usual cultivation media (SCA) which contains a combination of Casein and KNO_3 as nitrogen sources.

Table. 11: Diameter of zone of inhibition zone in mm of LABACT 21 grown on different nitrogen sources against *Shigella dysnetrea*.

Nitrogen sources	Test microorganisms and zone of inhibition in mm	
	S.dysentrea	Final pH
NoNitrogen source	0	9.81
Casein	8	9.48
Yeast extract	8	9.56
Peptone	8	9.60
Tryptone	8	9.49
$NaNO_3$	8	9.63
KNO_3	8	9.66
NH_4NO_3	8	9.30
$(NH4)_2SO_4$	8	9.57

N.B. measurement includes the diameter of the disc (6mm).

5.3.5. Effect of different salt concentration on growth and antibiotic production by LCHACT17 and LABACT21

The final dry biomass measurement indicated that both LCHACT17 and LABACT21 can grow in cultivation media from the cultivation media without NaCl up to 5% NaCl concentration. However for LCHACT 17 the highest weight was obtained from 2% NaCl and the lowest from 9% NaCl. On the other hand for LABACT 21 the highest weight was obtained from 0.5% NaCl and the lowest from 9% NaCl. These indicated the poor tolerance of both isolates on 9% NaCl concentration. (Figures.8 and 9).

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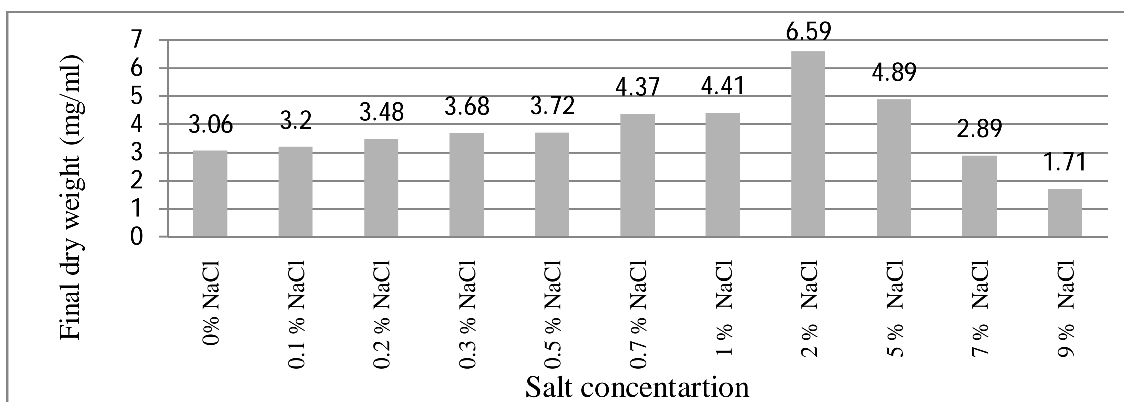

Figure. 8: Final dry biomass of LCHACT 17 on different NaCl concentration.

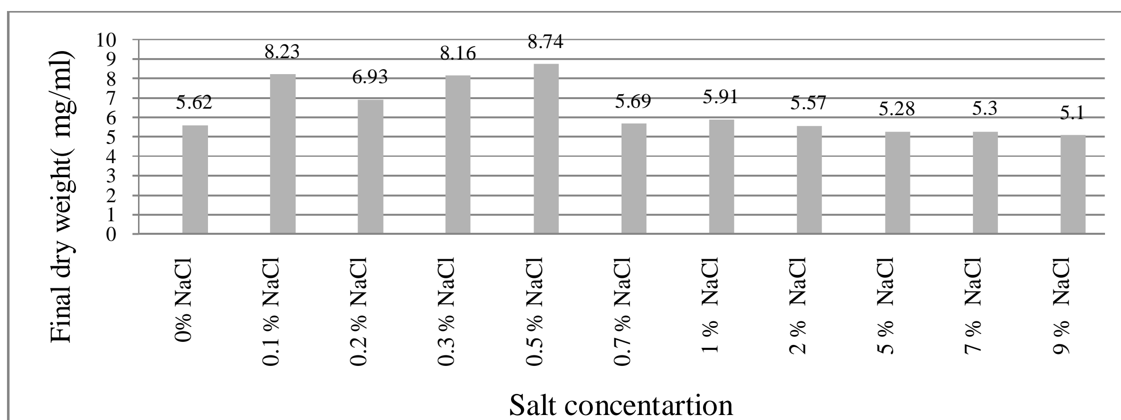

Figure.9: Final dry weight of LABACT 21 on different NaCl concentration.

As shown in Tables 12 and 13 below maximum antibacterial metabolite production was detected when the salt concentration was 1% against *Salmonella typhii* and *Streptococcus Pneumonia* (by LCHACT17) *Escherichia coli and Pseudomonas aeruginosa* (by LABACT21). (Appendix 2, Figure 5 A and B) On the other hand weak antibacterial activity was detected from the CF of both isolates grown on 5%, 7% and 9% NaCl concentration and no antibacterial metabolite from both isolates grown without NaCl.

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Table. 12: Diameter of zone of inhibition in mm of LCHACT17 grown in different NaCl concentration.

Salt(NaCl) Concentration	Test microorganisms and zone of inhibition in mm			
	P.aeruginosa	*S.typhii*	*S.pneumonia*	Final pH
No Nacl	0	0	0	9.59
0.1%	0	0	0	9.64
0.2%	0	0	0	9.56
0.3%	0	0	0	9.49
0.5%	9	0	0	9.66
0.7%	9	8	8	9.46
1 %	9	12	12	9.44
2 %	9	8	8	9.33
5%	8	0	8	9.01
7%	8	0	8	8.59
9%	8	0	0	8.71

N.B. measurement includes the diameter of the disc (6mm).

Table 13: Diameter of zone of inhibition in mm of LABACT 21 grown in different NaCl concentration.

Salt(NaCl) Concentration	Test microorganisms and zone of inhibition in mm			
	E.coli	*P.aeruginosa*	*S.typhii*	Final Ph
No Nacl	0	0	0	9.69
0.1%	0	0	0	9.97
0.2%	8	0	8	9.86
0.3%	8	0	8	9.85
0.5%	8	0	8	9.88
0.7%	8	0	8	9.85
1 %	9	10	8	9.62
2 %	0	8	8	9.58
5%	0	0	0	8.74
7%	0	0	0	8.63
9%	0	0	0	8.70

N.B. measurement includes the diameter of the disc (6mm).

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5.3.6 Effect of pH on growth and antibiotic production by LCHACT 17 and LABACT 21

Both isolates showed growth from pH 8- 12, but with maximum biomass between pH 9-11 at the 7th day. Poor growth at neutral pH and no growth at pH 13 were observed. (Figure. 10 and 11)

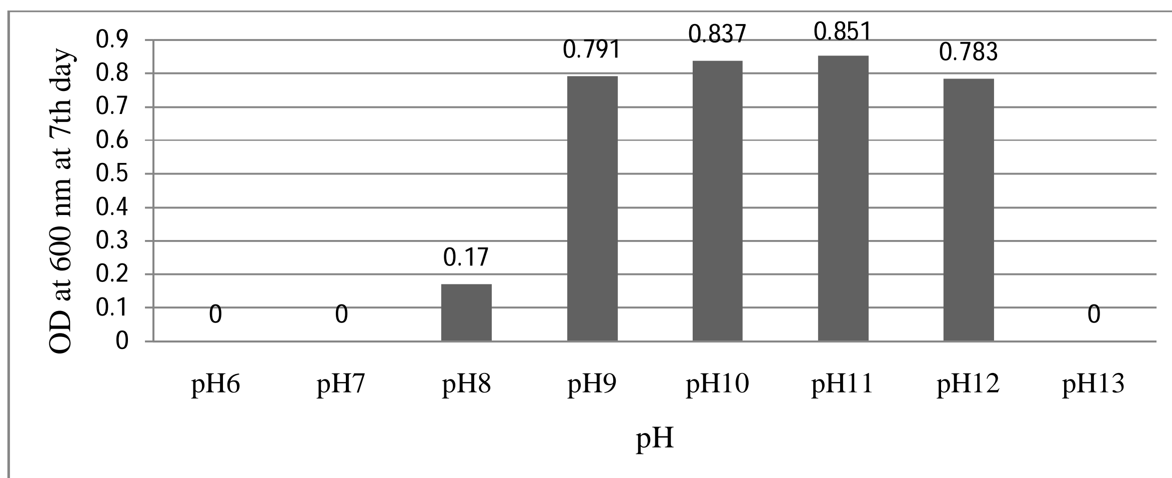

Figure. 10: Biomass of LCHACT 17 grown on different pH measured at 600nm using spectrophotometer.

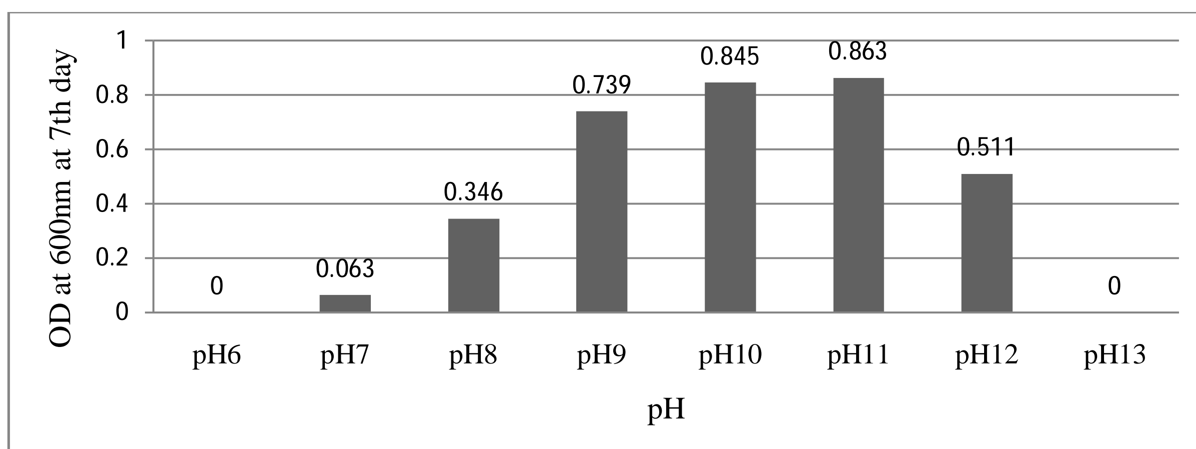

Figure.11: Biomass of LABACT 21 grown on different pH measured at 600nm using spectrophotometer.

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As shown in Tables 14 and 15 for both isolates antibacterial metabolite production was more favored when the pH of the cultivation media was adjusted between pH 9-11, with maximum activity detected at pH 11(for LCHACT 17) pH 10 (for LABACT 21).

Table.14: Diameter of zone of inhibition of LCHACT 17 grown on different pH

pH	Test microorganisms and diameter of zone of inhibition		
	S.aureus	*P.aeruginosa*	*S.pneumonia*
pH 6	0	0	0
pH 7	0	0	0
pH 8	8	0	0
pH 9	8	8	8
pH 10	8	8	9
pH 11	8	9	12
pH 12	0	8	8
pH 13	0	0	0

N.B. measurement includes the diameter of the disc (6mm).

Table.15: Diameter of zone of inhibition of LABACT 21 grown on different pH

pH	Test microorganisms and diameter of zone of inhibition(mm)		
	S.aureus	*P.aeruginosa*	*S.dysentrea*
pH 6	0	0	0
pH 7	0	0	0
pH 8	0	0	0
pH 9	8	9	8
pH 10	8	9	9
pH 11	8	9	9
pH 12	8	0	8
pH 13	0	0	0

N.B. measurement includes the diameter of the disc (which is 6mm).

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5.3.6.1. Final pH measurement

The final pH measured for both isolates grown on different cultivation media indicated that the pH remained alkaline regardless of the medium constituents. Hence production of antibacterial metabolite was favored when the culture condition was alkaline.

5.4. pH and temperature stability of the culture filtrate of LCHACT 17 and LABACT 21

5.4.1. pH stability of LCHACT17

As shown in Table 16. The culture filtrate of LCHACT 17 was stable at alkaline pH but weak stability at pH 12 and unstable at lower pH values.

Table.16: pH stability of the CF of LCHACT 17

LCHACT 17	Test microorganisms and zone of inhibition(in mm)	
	S.aureus	*P.aeruginosa*
pH2	0	0
pH4	0	0
pH6	0	0
pH8	9	8
pH10	9	8
pH 12	8	8
Untreated CF(pH9)	12	10

N.B. measurement includes the diameter of the disc (6mm).

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5.4.2. pH stability of LABACT 21

The culture filtrate of LABACT 21 was stable at alkaline pH values except weak stability at pH 12. On the other hand the CF was not stable at lower pH values (Table. 17.).

Table.17: pH stability of the CF of LABACT 21

LABACT 21	Test microorganisms and zone of inhibition(in mm)	
	P.aeruginosa	*S.dysentrea*
pH2	0	0
pH4	0	0
pH6	0	0
pH8	8	8
pH10	8	8
pH 12	8	0
Untreated CF(pH9.63)	10	8

N.B. measurement includes the diameter of the disc (6mm).

5.4.3. Temperature stability

The temperature stability of LCHACT 17 and LABACT 21 on *Streptococcus Pneumonia* and *Shigella dysentrea* respectively is given in Table 18 below. It was observed that the CF of LCHACT 17 was stable from 40°c -60°c while CF of LABACT 21 was stable only at 40°c.

Table. 18: Temperature stability of antibacterial metabolite of both isolates

Isolate	40°C	50°C	60°C	70°C
LCHACT 17	+	+	+	-
LABACT 21	+	-	-	-

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5.5. Determination of MIC

From the turbidity measurement, the higher dilutions which inhibit visible growth of the test pathogen (*Streptococcus Pneumonia*) was 2^{-3} dilutions thus the MIC of the antibacterial metabolite of LCHACT 17 against *Streptococcus Pneumonia* was 12.5mg/ml (Figure.12.).

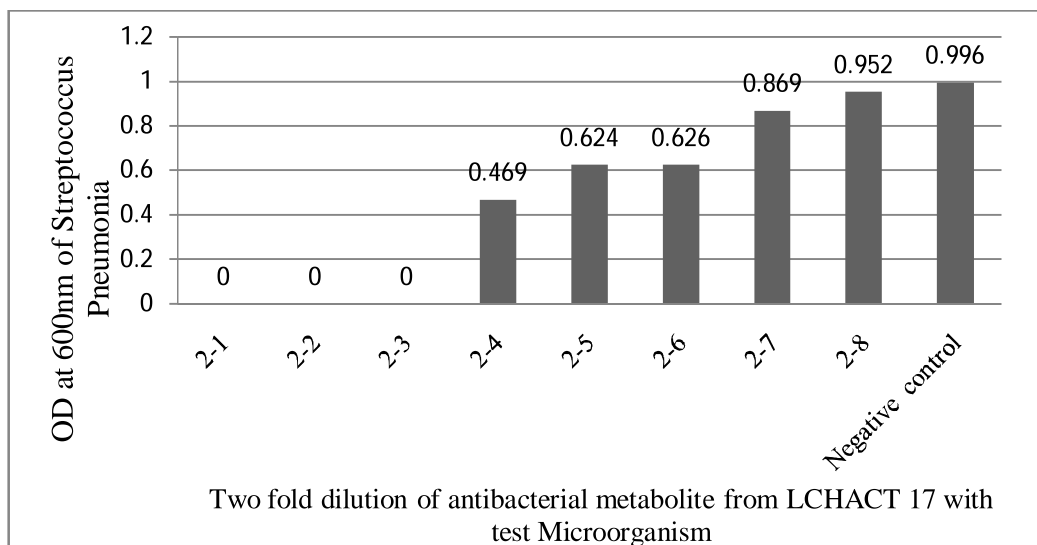

Figure.12: Minimum inhibitory concentration of antibacterial metabolite from LCHACT 17 against *Streptococcus Pneumonia.*

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6. Discussion

The results obtained from screening of alkaliphiles isolated from the three soda lakes for antibiotic production indicated that many of them produce antibacterial metabolites. This suggests the existence of antibiotic producing actinomycetes in those soda lakes. Biniam Wondale, (2008) also reported that some alkaliphillic actinomycetes isolated from Ethiopian soda Lakes were able to produce antibiotics against bacterial and fungal species.

Based on the results of morphological and biochemical tests indicated in Table.1 isolate LABACT 21 could be assigned to the genus *Streptomyces* as compared to the general property of this genus listed by Kämpfer, *et al*., 1991. The genus Streptomyces are gram positive, aerobic members of the order actinomycetales. Many strains produce a wide array of antibiotics, and pigment. The catalase reaction is positive, and generally, nitrates are reduced to nitrites. Most representatives are able to degrade polymeric substrates like starch and casein. On the other hand LCHACT17 could be assigned to the genus *Nocardia*. The genus *Nocardia* is Gram-positive, non-motile, catalase positive actinomycetes. They form rudimentary to extensively branched substrate hyphae that often fragment in situ or, on mechanical disruption, disrupt into rod shaped to coccoid, non motile elements. It has been indicated most *Nocardia* form carotenoid-like pigments. Soluble brown or yellowish diffusible pigments may be produced (Dworkin *et al*., 2006).

Isolate LCHACT17 showed antimicrobial activity against both Gram-positive and Gram-negative bacteria with variable activity on *Shigella dysentrea* when tested with crude culture filtrate and crude ethyl acetate extract. On the other hand isolate LABACT21 showed good antimicrobial activity against *Shigella dysentrea*. From all tested organisms *Shigella dysentrea* was relatively resistant to antibacterial metabolites from both isolates. Since *Shigella dysentrea* was a clinical isolate, it might have been exposed to different commercially available antibiotics which could probably induced for the development of antibiotic resistance.

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Effect of incubation period on growth and antibiotic production by LCHACT17 and LABACT21 indicated that high level antibiotic production (in terms of multiple inhibition and diameter of zone of inhibition) was obtained at 12th and 14th day of incubation respectively and production gradually showed decrement. (Tables.2 and 3.). Similarly Ripa *et al*., 2009 reported highest level antibiotic production by new Streptomyces species was obtained after 10 days of incubation and then production was declined gradually. In another report it has been indicated that production of antimicrobial metabolite by S*treptomyces* sp isolated from Natore, Bangladesh was started after 3 days of incubation. The highest level was obtained after 10 days of incubation and then production was declined gradually (Saha *et al*., 2010). In microbial cultures, the production of secondary metabolites and growth related functions do not occur simultaneously. Indeed the secondary metabolites accumulate only after the growth phase (tropophase) *i.e.* when the culture attains a specific growth rate (Nigam *et al*., 2007).

It was observed that for both of the isolates there was a relatively consistent increase of biomass with subsequent increment of phosphate concentration (Figures. 2 and 3)., due to the fact that phosphate is required for key biological functions like nucleic acid synthesis and for construction of phospholipids. Antibiotic production was more favored when phosphate concentrations was 0.2 % for both isolates whereas in higher phosphate concentration (above 0.2%) production was delayed or occurred at lower level in comparison with the lower concentration of phosphate. (Tables.4 and 6). It has been reported that Phosphate concentrations above 0.005% suppresses production of natamycin by *Streptomyces natalensis* (Farid *et al*., 2000). In line with this it has been reported that when phosphate concentration in the culture medium decreases below a threshold level, bacteria increase their production of a variety of metabolites that might serve as direct antagonists to other microorganisms (Vining, 1992).

Soluble starch, glycerol and, D-xylose were suitable carbon sources respectively than the rest for production of antibacterial metabolite by LCHACT17. (Table. 8.). On the other hand sucrose, glycerol, soluble starch and D-xylose, were suitable carbon sources than the rest for antibiotic production by LABACT 21. (Table.9). The results are comparable with Ripa *et al*., (2009) as no antibiotic was produced when the medium was supplemented with galactose, lactose, raffinose and maltose as a sole carbon source by a new streptomyces species. In addition Yang and Ling

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(1989) obtained similar results when they observed that a small amount of soluble starch or other fermentable polysaccharide was good for secondary metabolite production. On the other hand it was observed that in case of Streptomyces species, with regards to carbon sources species specific variation may occur for cell growth and secondary metabolites production (Jonsbu *et al*., 2002).

For isolate LCHACT17 among the nitrogen sources used yeast extract was suitable in the production of antibacterial metabolites that were active against *E. coli* and *S. aureus* at higher level followed by sodium nitrate, casein, and potassium nitrate that were effective against most of the test bacteria. (Table.10). These is due to the fact that yeast extract is rich in vitamins, minerals, amino acids, digested nucleic acids and other growth factors which could possibly induce antibacterial metabolite synthesis. The results are comparable with Ripa *et al*., (2009) as they indicate yeast extract followed by sodium nitrate; peptone and KNO_3 gave highest antimicrobial metabolite production by new Streptomyces species. In general antibacterial metabolite production can be influenced by the type of nitrogen source used in a cultivation media.

The highest biomass observed at 2% and 0.5% NaCl concentration for LCHACT17 and LABACT21 respectively and lower biomass at 7% and 9% (Figures.8 and 9)., by both isolates can be attributed to the preference of a cultivation media that resemble their natural environment. In fact the salinity level of Lake Abijiata and Lake Chitu were found to be 26g/l and 45g/l respectively (Elizabeth Kebede *et al*., 1994). These could contribute to the physiological tolerance limit of the microorganisms isolated from them near to these specified salinity condition. Similarly the high level antibacterial metabolite production obtained at 1% NaCl concentration and a decrease / absence at 5% and above could also be attributed to this fact. (Tables.12 and 13.). In fact Salt concentration has a profound effect on the production of antibiotic from microorganism due to its effect on the osmotic pressure to the medium (Pelczer *et al*., 1993).

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Both LCHACT17 and LABACT21 from Lake Chitu and Lake Aijiata respectively grew well at alkaline pH ranging from pH 9-11(Figures.10 and 11). Elizabeth Kebede *et al*., (1994) also reported that the pH of Lake Abijiata as 9.9 and of Lake Chitu as 10.2. Thus this growth at alkaline pH could be attributed to the suitability of the culture condition which mimics their natural environment. Production of antibacterial metabolite by LCHACT17 and LABACT21 at alkaline pH is comparable with some Streptomyces species recorded to produce antibiotics against bacteria, fungi and yeast at alkaline pH (Basilio *et al*., 2003).

Low and high pH levels were found to have a detrimental effect on stability of antibiotic metabolites and High temperatures were found to destabilize the antibiotics. Reduction of antimicrobial activity of antibiotic metabolites on subjection to different temperatures and pH levels has also been reported in other studies (Augustine *et al*., 2005). The MICs of the crude ethyl acetate extract of LCHACT17 against *Streptococcus Pneumonia* was 12.5 mg/ ml which indicates the active metabolite in the crude extract was existed at low concentration.

From the findings of this study it can be concluded that alkaliphillic actinomycetes from Ethiopian soda lakes should be taken in to consideration in the search of new antibacterial metabolites. In line with this Singh *et al*., (2009) suggested that apart from normal actinomycetes, the salt-tolerant and alkaliphillic actinomycetes are much less explored for their antimicrobial potentiality and it would be significant to pay more attention to extremophilic actinomycetes for new generation of antimicrobial agents.

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7. Conclusion

Based on the findings of this study the following conclusions were made

- § Alkaliphiles which exist in the soda lake habitats studied are capable of producing antimicrobial agents.
- § Productions of antimicrobial agents from those isolates were favored when the cultivation media was alkaline.
- § Antimicrobial production by both isolates was favored when phosphate concentration was low.
- § The highest production of antimicrobial metabolite by LCHACT17 was obtained when starch was used as the only carbon source, while for LABACT21 sucrose was preferred carbon source. Sodium nitrate and yeast extract (in terms of higher inhibition zone) favored better antimicrobial production by LCHACT 17 while combination of casein and potassium nitrate were suitable nitrogen sources for LABACT 21.
- § Different sodium chloride concentration has also a variable effect on antimicrobial agent production; 1% sodium chloride was favorable for antimicrobial metabolite production by both isolates.
- § The antimicrobial metabolites from both isolates were sensitive to lower pH (pH6 and below) and higher pH (pH 12 and above) values as compared to the untreated control.
- § Temperatures have adverse effect on stability of antimicrobial metabolites extracted from both isolates except with slight tolerance of LCHACT 17 up to 60°c.

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8. Recommendations

Based on the findings of this study the following recommendations are suggested:

- In the future identification and purification of the bioactive compounds isolated from those alkaliphiles could be further tested.

- The diversity of the alkaliphillic actinomycetes could be further studied.

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Appendices

Appendix. 1

Antibacterial activity of LCHACT 17 and LABACT 21 grown on the same condition, on starch casein agar media using culture filtrate at 13th day.

Isolates	Test microorganisms and inhibition zone in mm					
	E. coli	*S. aureus*	*P.aeruginosa*	*S.dysentrea*	*S.typhii*	*S.pneumonia*
17(1)	9	9	10	0	8	9
17(2)	9	9	9	0	8	10
21(1)	10	12	9	8	9	9
21(2)	10	11	9	8	9	9

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Appendix 2. Antibacterial activity of alkaliphilic actinomycetes on different test organisms

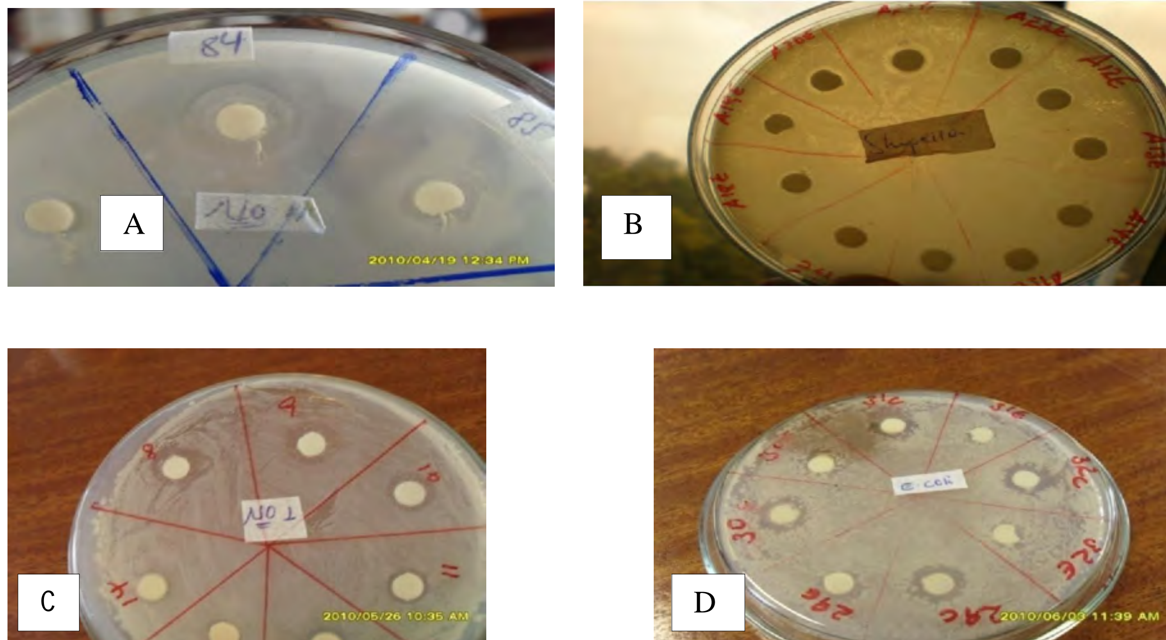

Figure 1. Antibacterial activity of representative alkaliphiles isolated from the three Lakes, (A). Crude ethyl acetate extract of LCHACT 84 and 85 from Lake Chitu against *Salmonella typhi*. (B) Crude ethyl acetate extract of LABACT 21 from Lake Abijiata against *Shigella dysentrea*. (C and D). Crude ethyl acetate extract and cell free culture supernatant of LSHACT 8, 9, 10, 11, 12, 13, 14, 29, 30, 31 and 32 from Lake Shalla against *Escherichia coli*.

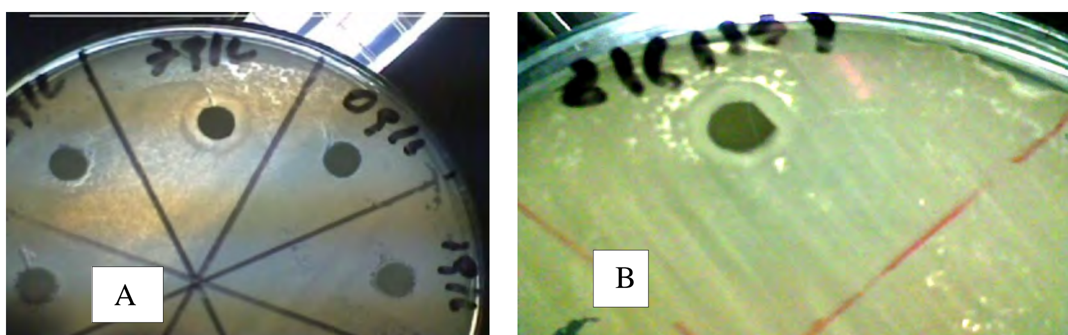

Figure.2. Antibacterial activity of LCHACT 17 grown at 0.7 % phosphate concentration against *Streptococcus pneumonia* (12th day CF) (A) and LABACT 21 grown on 0.2% phosphate concentration against *Staphylococus aureus* (B) (14th day CF).

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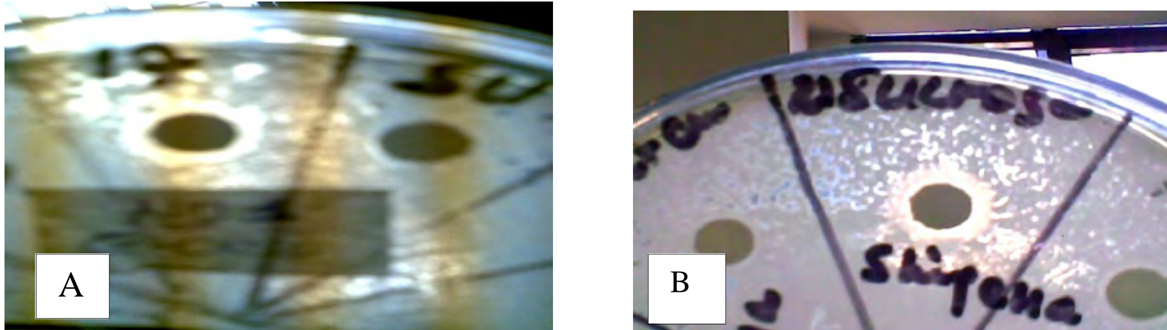

Figure.3: Antibacterial activity of LCHACT 17 grown on soluble starch against *Escherichia coli*(A) and antibacterial activity of LABACT 21 grown on sucrose against *Shigella dysentrea* (B).

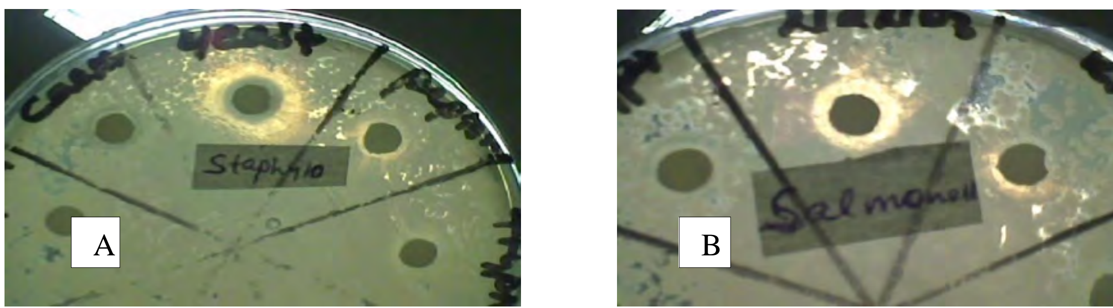

Figure. 4: Antibacterial activity of LCHACT 17 grown on yeast extract against *Staphylococcus aureus*(A) and LCHACT 17 grown on $NaNO_3$ against *Salmonella typhii* (B).

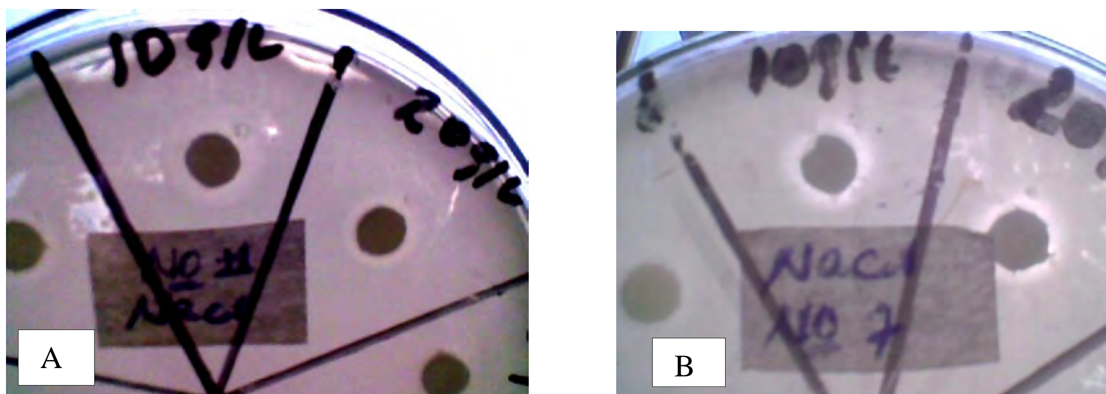

Figure.5: Antibacterial activity of LCHACT 17 grown on 1% and 2% NaCl concentration against *Salmonella typhii* (A) and of LABACT 21 against *Pseudomonas aeruginosa*(B).

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Declaration

I, the undersigned, declare that this thesis is my original work and has not been presented for any degree in any other University. All sources of materials used for the thesis have been accordingly acknowledged.

Name of Candidate: Daniel Mehabie

Signature :

Date: July, 2011