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Transformation of *Lactobacillus acidophilus* with Hygromycin Gene Originated from *Streptomyces hygroscopicus*

A Dissertation

Submitted to the Council of College of Science / Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Doctorate of Philosophy in Biotechnology

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الأهداء

الى من تَحمِلُني بين يديها ... دعاءٌ متصلٌ الى السماء
قطرةٌ في بحركِ العظيم ... حباً وطاعةً وبراً

أليكِ أُمي

الى من سأظل أفتقدهُ في مواجهة الصعاب
القلبُ الناصعُ بالبياض... عنوان كبريائي وفخري

أبي رحمهُ الله

الغائب الحبيب ...الحاضرُ في قلبي ماحييت

أخي الشهيد

الى شموعٍ مُتقده أنارت ظلام حياتي دوماً

أخواتي

الى يدٍ بيضاء ... لم تَخذلني يوماً

د. عمار الزبيدي

اليهم جميعاً أُهدي ثمرة جهدي المتواضع هذا

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Summary

To Investigate the ability of *Streptomyces* isolate to produce hygromycin B antibiotic as antithelmantic agent in poultry feed and selecting a suitable expression vector encoded hygromycin B phosphotransferase resistance gene and transforming *Lactobacillus* isolateby electroporation , from a total of 53 *Streptomyces* isolates, DNA was extracted and screened for the presence of hygomycin B gene using specific designed primer sets in Polymerase Chain Reaction (PCR) technique. Only three of the isolates were positive for the existence of such a gene which is responsible for the production of this antibiotic. To select the most efficient one, the three isolates were subjected to the antimicrobial activity evaluation. Then, the selected isolate was identified and characterized morphologically and biochemically.

The gene of hygromycin B phosphotransferase (*hph*) was also detected in the *Streptomyces* isolate using specific designed primers. When the hygromycin B was extracted by ethyl acetate, which separates organic phase from aqueous phase in the broth culture filtrate, only the aqueous phase showed a significant antimicrobial activity by using agar well diffusion technique. At a concentration of 25mg/ml (as crude extract), this phase excreted its activity against the test microorganisms which include; one G(+) bacteria (*Staphylococcus aureus*), five G(–) bacteria (*Pseudomonas aeruginosa , Proteus mirabilis, Escherichia coli , Klebsiella pneumoniae*, *Salmonella typhi*) and one yeast (*Saccharomyces cerevisiae*).

After detecting the aminoglycoside hygromycin B by the Thin Layer Chromatography (TLC) method to ensure presence of the antibiotic, the same flow rate (R_f) value (0.357), as that of the standard hygromycin B ,was obtained.

Results of the optimization conditions showed that the highest antimicrobial activity of hygromycin B was obtained at a medium pH of 8 and incubation temperature of 35°C for 10 days. When the toxicity of hygromycin B crude extract under such conditions was examined on mice liver, mild effects appeared.

Lactobacillus acidophilus isolated from a yoghurt sample was identified conventionally, by API 50 kit, and also by using 16S ribosomal gene technique. Cloning vector containing the (*hph*) gene was extracted from *E.coli* bacteria and used to transform cells of *Lactobacillus acidophilus* by using electroporation technique,that give transformation efficiency of $1.75x10^{8}$ cfu/100ng of plasmid DNA then the transformed cells were tested by specific *hph*–designed primer and give positive result in gel electrophoresis technique.

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List of Abbreviations

GRAS	**Generally Recognize As Safe**
Hm	**Hygromycin B**
LAB	**Lactic Acid Bacteria**
HPH	**Hygromycin Phosphotransferase**
MCSs	**Multiple-Cloning Sites**
LB	**Luria–Bertani**
TE	**Tris- EDTA**
PCR	**Polymerase Chain Reaction**
NCBI	**National Center for Biotechnology Information**
ISP	**International *Streptomyces* Project**
TLC	**Thin Layer Chromatography**
EDTA	**Ethylene Diamine Tetraacetic Acid**
Rf	**Retardation factor**
TBE	**TRis – Borate – EDTA**
TAE	**Tris – Acetate - EDTA**

CHAPTER ONE
INTRODUCTION
AND
LITERATURE REVIEW

1.1 Introduction:

Streptomyces includes Gram positive, high GC-content, sporulating bacteria found predominantly in soil. *Streptomyces* is characterized by its complex secondary metabolism for producing antibiotic compounds and other metabolites with medicinal properties. Genomic studies, genomic mining and biotechnological approaches have been employed in the search for new antibiotics and other drugs (Anita *et al.*, 2004).

Hygromycin B is an unusual antibiotic in that it is active against both prokaryotic and eukaryotic cells and specifically it inhibits protein synthesis at the translocation step. It is produced by *Streptomyces hygroscopicus* which possesses a unique hygromycin B -phosphotransferase (HPH) that fully inactivates the antibiotic by phosphorylation of the 7"-hydroxyl group of the destomic acid moiety thus being a new type of aminocyclitol-O-phosphotransferase. *S. hygroscopicus* apparently requires only the HPH activity to provide resistance to hygromycin B (Zalacain *et al.*, 1987)

Hygromycin B is a trisaccharide aminoglycoside with a terminal cyclitol, reported by researchers in the early 1950s. It belongs to the destomycin family of aminoglycosides which contain a unique cyclic structure linking the terminal saccharide. Hygromycin B exhibits broad spectrum antibiotic and anthelmintic activity, and is used as a feed additive for some animal meats like poultry as anti-worming agent in a product name (Hygromix). Hygromycin B inhibits polypeptide synthesis by binding peptidyl-tRNA and preventing translocation by Elongation Factor 2 (Burgett *et al.*, 1983).

Lactic acid bacteria, including *Lactobacillus* species, can serve a dual function by acting as agents for food fermentation and, in addition, potentially imparting health benefits. Species of *Lactobacillus* and *Bifidobacterium* are most commonly used as probiotics, but the yeast *Saccharomyces cerevisiae* and some *E. coli* and *Bacillus* species are also used as probiotics (Guarner *et al.*, 2008).

Lactobacillus genus, which belongs to the family Lactobacillaceae, contains lot of different species. They are "friendly" bacteria that normally live in our digestive, urinary, and genital systems without causing disease. (Goyal *et al.,* 2012).

Lately, the interest in developing new and genetically engineered lactic acid bacteria (LAB) strains with improved properties has been continuously growing. *Lactobacillus* and many others are considered as probiotic bacteria that have the ability to efficiently secrete improved or novel recombinant proteins directly into the culture medium of medical and industrial biotechnology application (Spath *et al*., 2012). Several vector systems exist and different plasmid backbones and promoters are available for cloning and protein expression in LAB. Often, such approaches are based on the generation of a great variety of genetically different clones and the subsequent screening for the desired phenotype (Okano *et al.,* 2010).

During the last decades many transformation protocols for LAB strains have been published. The successful introduction of plasmid DNA into LAB is dependent on strain specific features such as cell wall structure, composition of plasmid size and the origin of replication. In some LAB, the low number or even lack of transformants obtained after electroporation, may be attributed to various restriction modification (RM) systems encoded by the host. RM systems are widely spread in bacteria and serve the protection of invading DNA such as foreign plasmids or the DNA of bacteriophages. Most of these systems consist of a restriction enzyme and a corresponding methyl- transferase that blocks the restriction activity, thus, protects the genome from self cleavage (type I , II and III RM systems) (Blumenthal and Cheng ,2002).

Since no natural system of competence to take up DNA has been found in the genus, electroporation is a technique that has found application in the transformation and transfection of many types of eucaryotic cells including plant and yeast cell protoplasts, and B lymphocytes. Electroporation uses a high-voltage

electric discharge through a suspension of cells to induce transient 'pores' in the cell membrane through which DNA enters the cells (Chassy and Flickinger, 1987).

This study aimed to:

1- Identification of *Streptomyces* isolate producing hygromycin B antibiotic
2- Molecular detection of gene responsible for hygromycin B production.
3- Optimize the production of hygromycin B.
4- Estimate the toxicity of the antibiotic.
5- Isolate and identify *Lactobacillus acidophilus*.
6- Selecting a suitable expression vector encoded hygromycin B resistance gene and transforming *Lactobacillus* isolate by electroporation to improve the antimicrobial activity of *Lactobacillus* as probiotic bacteria .

1.2 Literature review:

1.2.1 Probiotic

1.2.1.1 Definition and history:

Probiotics are a live microorganisms that can be formulated into many different types of products, including foods, drugs, and dietary supplements provide benefits to health when consumed(Francisco *et al.*, 2008). The term probiotic is currently used to name ingested microorganisms associated with beneficial effects to humans and animals. Introduction of the concept is generally attributed to Nobel Prize recipient Eli Metchnikoff, who in 1907 suggested that "the dependence of the intestinal microbes on the food makes it possible to adopt measures to flora modification in our bodies and to replace the harmful microbes by useful microbes" (Metchnikoff, 1907).

Probiotics must be safe for their intended use. The 2002 FAO/WHO guidelines recommend that, though bacteria may be Generally Recognized as Safe (GRAS), the safety of the potential probiotic should be assessed by the minimum required tests:

- Determination of antibiotic resistance patterns.
- Assessment of certain metabolic activities (e.g., D-lactate production, bile salt deconjugation).
- Assessment of side-effects during human studies.
- Epidemiological surveillance of adverse incidents in consumers (post-market).
- If the strain under evaluation belongs to a species that is a known mammalian toxin producer, it must be tested for toxin production. One possible scheme for testing toxin production has been recommended by the EU Scientific Committee on Animal Nutrition (Scan, 2000).
- If the strain under evaluation belongs to a species with known hemolytic potential, determination of hemolytic activity is required.

Finally probiotics have to be supplied in adequate amounts which may be defined as the amount able to trigger the targeted effect on the host. It depends on strain specificity, process and matrix, as well as the targeted effect.

Probiotic is a relatively new word meaning 'for life', which is used to name microorganisms that are associated with the beneficial effects for humans and animals. These microorganisms contribute to intestinal microbial balance and play a role in maintaining health. The probiotic microorganisms consist mostly of the strains of the genera *Lactobacillus* and *Bifidobacterium*, but strains of *Bacillus*, *Pediococcus* and some yeasts have also been found as suitable candidates. Together they play an important role in the protection of the organism against harmful microorganisms and also strengthen the host's immune system. Probiotics can be found in dairy and non-dairy products. They are usually consumed after the antibiotic therapy (for some illnesses), which destroys the microbial flora present in the digestive tract (both the useful and the targeted harmful microbes). Regular consumption of food containing probiotic microorganisms is recommended to establish a positive balance of the population of useful or beneficial microbes in the intestinal flora (*Carlos et al., 2010)*.

As shown in table (1.1), various microorganisms are used as probiotics but the mostly used are bacteria belonging to the species of *Lactobacillus* and *Bifidobacterium* (Anuradha and Rajeshwari, 2005).

Table (1-1) : Microorganisms used as probiotics

A. Bacteria: i. *Lactobacillus: acidophilus, sporogenes, plantarum, rhamnosum, delbrueck, reuteri, fermentum, lactus, cellobiosus, brevis* ii. *Bifidobacterium*: *bifidum, infantis, longum, hermophilum, animalis* iii. *Streptococcus*: *lactis, cremoris, alivarius, intermedius* iv. *Leuconostoc* v. *Pediococcus* vi. *Propionibacterium* vii. *Bacillus* viii. *Enterococcus* ix. *Enterococcus faecium*
B. Yeast and molds: *Saccharomyces cerevisiae; Aspergillus niger, A. oryzue, C. Pintolopesii, Sacharomyces boulardii.*

1.2.1.2 Characteristics of probiotics:

It should be a strain, which is capable of exerting a beneficial effect on the host animal, e.g. increased growth or resistance to disease. It should be also non-pathogenic and non-toxic resistance to low pH and organic acids (Fuller, 1989).The culture should adhere to the intestinal wall and proliferate (Berent *et al.*, 1994; Ouwehand *et al*., 1999). The potency of probiotic was loosed when they are exposed to oxygen, moisture, and heat. For that reason, probiotic supplements should be freeze-dried, nitrogen-packaged and refrigerated to maintain their potency and viability (Goldin and Gorbach, 1984).

1.2.1.3 Mechanisms of action of probiotics:

Probiotic bacteria have multiple and diverse influences on the host. Different organisms can influence the intestinal luminal environment, epithelial and mucosal barrier function, and the mucosal immune system. They exert their effects on numerous cell types involved in the innate and adaptive immune responses, such as epithelial cells, dendritic cells, monocytes /macrophages, B cells, T cells, including T cells with regulatory properties, and NK cells. Table (1-2) provides a simplified illustration of the main mechanisms of action of probiotics (Zhang *et al.*, 2007; Neurath, 2007).

Table (1-2) Mechanisms of action of probiotics

Antimicrobial Activity	Decrease luminal pH Secrete antimicrobial peptides Inhibit bacterial invasion Block bacterial adhesion to epithelial cells
Enhancement of Barrier Function	Increase mucus production Enhance barrier integrit
Immunomodulation effects on:	Epithelial cells Dendritic cells Monocytes/macrophage Lymphocytes - B lymphocytes - NK cells - T cells - T cell redistribution

1.2.2 Genus *Lactobacillus*:

The genus *Lactobacillus* belongs to the Phylum Firmcutes, Class Bacilli, Family Lactobacillaceae. *Lactobacillus*, also called Döderlein's bacillus, is a genus of Gram-positive facultative anaerobic or microaerophilic ,rod-shaped bacteria with a regular dimension of 0.5-1.5x10nM , single , paired , or small chain , catalase negative , and stable in acid media and salt with low G+C content (Makarova *et al.*, 2006; Stamer, 1976). They are a major part of the lactic acid bacteria group, named as such because most of its members convert lactose and other sugars to lactic acid (Dicks *et al.*, 2000). Many species are prominent in decaying plant material. The production of lactic acid makes its environment acidic, which inhibits the growth of some harmful bacteria. Several members of the genus have had their genome sequenced (Ljungh et *al.*, 2009).

With a long application history, lactobacilli are important microbes in industry, which contribute to the production of cheese, yogurt, and other fermented products. Apart from being isolated from a broad range of nutrient-rich environments (Henri et *al.*, 2008; Najjari *et al.*, 2008), lactobacilli are also part of the commensal human microbiota. They are abundant in the vagina (Martin *et al.*, 1999), and are also found in the oral cavity (Hojo *et al.*, 2007), the small intestine and the large intestine (Eckburg *et al.*, 2005). Because of their history of safe use and their natural presence in the human intestinal tract, commensal lactobacilli offer considerable potential as probiotics.

Even more than the other lactic acid bacteria (LAB), *Lactobacillus* species vary greatly in their phenotype, and scientists are faced with an unusually high level of phylogenetic diversity (Claesson *et al.*, 2007).

Colonization adhesion or persistence is an expected, but possibly not essential feature for strains being developed as probiotics. However, strains which have probiotic characteristic, are expected to have a wider impact on intestinal ecology, which may include production of antimicrobial compounds, and usually

requires survival in the stressful environment of the gastrointestinal tract, including resistance to acidic medium, and bile salts. The identification of genes or clusters that contribute to these probiotic-related characteristics is a challenge for scientists. Manipulation and genetic analysis of these bacteria will be essential to understand the function of probiotic and to optimize their performance *in vitro* and *in vivo*. More genetic analysis study of *Lactobacillus* species will increase the understanding of the probiotic characteristics of these strains. The development of genetic tools for lactic acid bacteria, especially commensal lactobacilli, has lagged significantly, notwithstanding a number of significant modern projects. Therefore, novel genetic tools are urgently essential to understand the biological possibility of many *Lactobacillus* species (Fang *et al.*, 2009).

1.2.2.1 *Lactobacillus acidophilus*

Lactobacillus acidophilus is a homofermentative species, fermenting sugars into lactic acid, and growing readily at rather low pH values (below pH 5.0) It has an optimum growth temperature of around 37 °C (Bâati . *et al.*,2000). *L. acidophilus* occurs naturally in the human and animal gastrointestinal tract and mouth. Some strains of *L. acidophilus* may have probiotic characteristics. These strains are commercially used in many dairy products, sometimes together with *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* in the production of acidophilus-type yogurt (Ljungh and Wadström , 2006).

Studies report few side effects from *L. acidophilus* when used at recommended doses. Some experts recommend limiting the daily dose to fewer than 10 billion living *L. acidophilus* organisms to reduce the side effects (Adolfsson *et al.*, 2004). Oral supplements of *L.acidophilus* in human have resulted in synthesis of B-complex vitamins and adsorption of calcium amilelioration of diarrhea and constipation and immunity activation (Reddy *et al.*, 1983).

1.2.3 *Lactobacillus* identification tools:

Identification of *Lactobacillus* species has been historically contentious, and some high-profile strains have been reclassified. Meaningful biological investigations of commensal lactobacilli benefit from robust identification and phylogenetic positioning of strains that are often isolated from very complex environments. The classification of lactobacilli is complex and controversial: Identification of commensal lactobacilli is difficult as they belong to a large extremely diverse genus *Lactobacillus* which contains 147 recognized species (Euzeby, 2008). The classic approach namly employment of API 50 CH strips (bioMérieux, Inc., Marcy l'Etoile, France) (Nigatu, 2000) which is based on carbohydrates fermentation profiles is common and efficient for the identification of lactobacilli.

Complicating identification are persistent errors in classification; for example, it is not robust to divide some species into subspecies (Li. *et al*., 2006) either by molecular techniques, or by reference to carbohydrate utilization profiles in the API 50 CH database. Therefore, the differentiation of *Lactobacillus* species according to physiological and biochemical criteria alone can be unreliable. Molecular approaches are more reliable, and have been used to distinguish lactobacilli at the species or strain level. Most of these molecular approaches are based on analyzing differences in PCR amplicons derived from fragments or regions including 16S ribosomal RNA (rRNA) gene, 16S-23S rRNA intergenic spacer region (Kwon *et al.*, 2004), *groEL* (*hsp*60 PCR-RFLP) (Blaiotta. *et al.*, 2008) or random DNA (randomly amplified polymorphic DNA, RAPD) (Johansson. *et al.,* 1995). These strain identification tools are efficient and can distinguish lactobacilli at species levels.

1.2.4 Actinomycetes:

Actinomycetes is a divers and a large group of Gram positive filamentous and or branching bacilli. They form mycelia, of a single kind, designated as a substrate

or vegetative or of two kinds, substrate and aerial. This form of microorganism reproduced by fission or by means of special spores or conidia, partially acid-fast, a branched bacteria has many microbiologic characteristics in common with members of the genera *Mycobacterium* and *Corynebacterium* (Waksman, 1967).

The G+C content of *Actinobacteria* (the new name for Actinomycetes) can be as high as 70%, though some may have a low G+C content (Ghai *et al.*, 2012).

The term aerobic actinomycetes is an informal designation for bacteria that belong to the order Actinomycetales. Historically, the actinomycetes were called the ray fungi and were thought to be related to the true fungi, such as, bread molds, because they formed mats (mycelia) of branching filaments (hyphae). However, unlike the true fungi, the actinomycetes have thin hyphae (0.5–1.5 micrometers in diameter) with genetic material coiled inside as free DNA. The cell wall of the hyphae is made up of a cross-linked polymer containing short chains of amino acids and long chains of aminosugars . These microorganisms are now recognized as true bacteria that are aerobic Michaeland June,1994).

A majority of the actinomycetes feed on protein or nonprotein organic matters. Actinomycetes live under the most diverse conditions, aerobic and anaerobic, and many actinomycetes are used to produce antibiotics, vitamins, pigments, amino acids, and other biologically active substances. Although the aerobic actinomycetes are infrequently encountered in clinical practice, they are important potential causes of serious human and animal infections (Habiba *et al.*, 2013).

Actinomycetes are susceptible to a wide range of antibiotics that are used to treatbacterial diseases, such as penicillin and tetracycline. Actinobacteria, especially *Streptomyces* sp., are recognized as the producer of many bioactive metabolites that are useful to humans in medicine, such as antibacterials (Mahajan,

2012), antifungals (Gupte, 2002) antivirals, antithrombotics , immunomodifiers, anti-tumor drugs and enzyme inhibitors (Bressan, 2003 ; Atta, 2009).

1.2.4.1 Streptomycetes and *Genus Streptomyces*:

Strepromycetes produce approximately 75% of the commercially and medically useful antibiotics (Miyadoh, 1993). In the course of screening for new antibiotics, several research studies are now oriented towards the isolation of new *Streptomyces* species from different soil samples.

The morphological differentiation of *Streptomyces* involves the formation of a layer of hyphae that can differentiate into a chain of spores. This process is unique among Gram-positives, requiring a specialized and coordinated metabolism (Omura *el al.,* 2001; Pantzer, and Volkmar, 2010)

The way of living of *Streptomyces*, is in many ways strikingly similar to that of filamentous fungi: both grow as branching hyphae that form a vegetative mycelium and disperse through spores that form on specialized reproductive structures called aerial hyphae, which emerge from the colony surface into the air. Another characteristic is of the genus is complex multicellular development , in which their germinating spores form hyphae, with multinuclear aerial mycelium, which forms septa at regular intervals, creating a chain of uninucleated spores. These similarities presumably are the result of adaptations to similar ecological niches, although the underlying mechanisms have different evolutionary origins. Like fungi, most Sreptomycetes live as saprophytes in the soil, although *Streptomyces* spp. also successfully inhabit a wide range of other niches, both terrestrial and aquatic, and some strains are plant and animal pathogens. The unsurpassed richness and diversity of the secondary metabolism of Streptomyces has made these organisms valuable providers of antibiotics and other bioactive molecules,and the production of these compounds is coordinated with the *Streptomyces* developmental programme (Hopwood , 2007 and Ohnishi , *et al.,* 2008) .

Streptomyces is the largest genus of Actinobacteria and the type genus of the family Streptomycetaceae (Kämpfer and Peter, 2006). Its species are Gram positive soil-dwelling filamentous bacteria with a complex cycle of morphological differentiation (Claessen *et al*, 2006). More than 500 *Streptomyces* species and subspecies have been described, the largest number of any bacterial genus (Eusebia JP, 2008).

Streptomyces members are potential sources for secondary types of bioactive metabolites possessing a variety of biological activities, including antimicrobial activity, which is used for human and animal treatment and act as anti-viral and anti-cancer compounds and in agriculture fields as herbicides, insecticides , antiparasitic compounds , anti-hypertensives, and immunosuppressives . It is estimated that this bacteria synthesizes more than 7,000 metabolites (Watve *et al.*, 2001; Bérdy, 2005).

Numerous classifications were devised to accommodate the increasing number of *Streptomyces* species, most of them based on a few subjectively chosen morphological and pigmentation properties which were rarely studied under standardized growth conditions (Atalan *et al.*, 2000). Biochemical, nutritional and physiological characters was also been used in Streptomycetes taxonomy, but usually was applied to only selected species (Kutzner *et al.*, 1989). The genus *Streptomyces* was classified in the family Streptomycetaceae that also includes a number of other taxa (Waksman, 1961).

1.2.4.2 *Streptomyces hygroscopicus*

Streptomyces hygroscopicus is a bacterial species in the genus *Streptomyces*. Gram-positive, aerobic, filamentous, biofilm-forming, rod prokaryote (bacterium). *Streptomyces* spp. belong to the Actinomycetes group and are bacteria that share many characteristics with the fungi. They grow usually as filaments (chains of cells) and often branch to form a network of filaments (mycelium) in the soil. At the tips of filaments spore chains of indefinite length develop . These soil bacteria are responsible for the musty odor of soil. This strain of *Streptomyces hygroscopicus* produces the antibiotic, hygromycin B, It is also a biofilm forming bacterium. Biofilms are primarily accumulations of bacteria in aqueous environments. They form when bacteria secrete slimy, mucilaginous materials that provide the microorganisms with a means of attachment to moist surfaces. Biofilm microorganisms carry out a variety of detrimental or beneficial reactions under certain environmental conditions **(**Backus *et al.*, 1954).

1.2.5 Hygromycin (Hm) B:

Hygromycin B is an aminoglycoside antibiotic produced by *Streptomyces hygroscopicus* that active against bacteria, fungi and higher eukaryotic cells by inhibiting polypeptide synthesis , It stabilizes the tRNA-ribosomal acceptor site, thereby inhibiting translocation (McGuire and Pettinger, 1953)**.**

It has been reported to interfere with translocation1 and to cause mistranslation at the 70S ribosome (Gonzales, A. *et al*. 1978; Singh, A. *et al*. 1979)**.**

Hygromycin B was originally developed in the 1950s for use with animals and is still added into chicken feed as an anthelmintic or anti-worming agent (product name: Hygromix).. Resistance genes were discovered in the early 1980s (Davies *et al.*, 1983; Burgett et *al.*, 1983). Hygromycin B show in figure (1-1) has a chemical formula $C_{20}H_{37}N_3O_{13}$ and molecular mass 275.53g/mol.

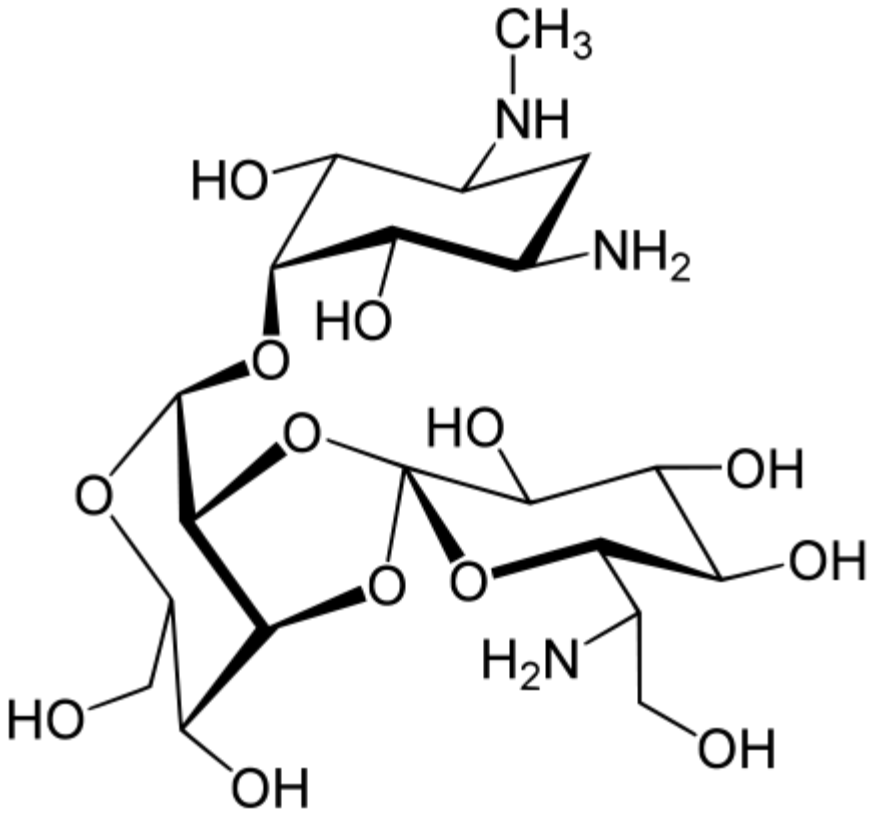


Figure 1-1 :- Chemical structure of Hygromycin B.(Borovinskaya *et al*.,2008)

1.2.5.1 Mechanism of action of hygromycin B:

In research it is used for the selection and maintenance of prokaryotic and eukaryotic cells that contain the hygromycin resistance gene. A more detailed examination of the mechanism of action has suggested that Hygromycin B also interferes with the translocation step in cell-free systems from both bacteria (Cabanas *et al.*, 1978) and yeasts (Gonzalez *et al.*, 1978). Most of the aminocyclitol antibiotics can be inactivated by at least one of three enzymatic mechanisms: (i) acetylation, (ii) adenylylation, or (iii) phosphorylation. Enzymes carrying out these reactions and the genes that code for them have been isolated from bacteria resistant to aminocyclitols (Davies and Smith, 1978.) and from *Streptomyces* species that produce these antibiotics (Leboul and Davies, 1982). Haas and Davies (1981) pointed out that the presence of these enzymes in bacteria usually makes the bacteria resistant to the appropriate antibiotic. Although

resistance to Hm has been found in the Hm-producing organism *Streptomyces hygroscopicus* there has been no previous report of plasmid-encoded resistance to this antibiotic in enterobacteria. Moreover, none of the numerous aminoglycoside-modifying enzymes reported to date has been shown to use Hm as a substrate.

This aminocyclitol antibiotic specifically blocks the translocation step on both 70s and 80s ribosomes (Cabaiias *et al.,* 1978; Gonzilez *et al.,* 1978). The drug also induces misreading both in vivo and in vitro and therefore promotes phenotypic suppression (Singh *et al.,* 1979). *S. hygroscopicus* contains a phosphotransferase (HPH) activity which phosphorylates hygromycin B, thus potentially providing the producing organism with autoimmunity against the toxic effects of the drug (Leboul & Davies, 1982). This interpretation appears to be valid since the gene (hyg) encoding the HPH activity has now been cloned in *S. lividans* and cells containing it became resistant to hygromycin B (Malpartida *et al.,* 1983). Such an enzymic inactivation is one of several ways by which antibiotic-producing *Streptomyces* spp. become resistant to their own secondary metabolites**.**

1.2.5.2 hygromycin phosphotransferase:

The hygromycin phosphotransferase (denoted *hpt, hph* or *aph*IV) gene was originally derived from *Escherichia coli*. The gene codes for hygromycin phosphotransferase (HPT), which detoxifies the aminocyclitol antibiotic hygromycin B. A large number of plants have been transformed with the *hpt* gene and hygromycin B has proved very effective in the selection of a wide range of plants, including monocotyledonous. Most plants exhibit higher sensitivity to hygromycin B than to kanamycin, for instance cereals. Likewise, the *hpt* gene is used widely in selection of transformed mammalian cells(Rao *et al.,* 1983). Leboul and Davies (1982) described the isolation of a gene from *Escherichia coli* that codes for Hm phosphotransferase activity and confered resistance to Hm,

1.2.6 Gene transfer:

No substance is important as deoxyribonucleic acid (DNA) because it carries within its structure the genetic information that determines the structures of protein which is a basic molecule for life. Importance and contribution to the biotechnology and molecular biology come from the genetic engineering. The efficiency and adeptness to introducing of exogenous DNA into organisms and their expression have been carried out. Transgenic plants and animals can be acquired by the introduction of exogeneous DNA molecule into targeted plants and animals with stable expression. The transferring method of DNA into organisms differs from organism to organisms. Generally, there are two DNA transferring approaches (Khan, 2010):

The first, involves the transfer of DNA by natural methods including; (Bacterial transformation, Conjugation, Transposition, Phage transduction, Retroviral transduction, Agrobacterium mediated transfer.

The second the transfer occurs by artificial methods including;

A) Physical methods (Microinjection, Macroinjection, Biolistics transformation, Protoplast fusion.

B) Chemical methods Transfer of DNA by use of polyethene glycol, DNA transfer by calcium phosphate, DNA transfer by calcium phosphate, Using DEAE-Dextran for DNA transfer, Liposome mediated transfer.

C) Electrical methods (Electrofusion, Electroporation).

Although genetic manipulation of strains for usage in food preparation is forbidden in many jurisdictions, there is a great deal to be learned by studying *Lactobacillus* gene function in a heterologous background. Thus it is important to develop methods for transfering a gene between strains and model hosts.

1.2.7 Electroporation:

Electroporation is one of the important and essential method to introduce foreign molecules into biological components, such as living mammalian cells,

human tissues and bacterial cells that has considerable implications for biological approach and medical applications (Gehl , 2003).

Electroporation process uses an electrical pulse to produce transient pores in the plasma membrane and by which allowing macromolecules passing into the cells. It is an efficient and successful process to transfer DNA into cells. Microscopic pores are induced in biological membrane by the application of electrical current. These pores are known as electropores which allow the ions, molecules and water to pass from one side to anther in the biological membrane. The pores can be recovered only if a suitable electric pulse is applied. The cell can recover spontaneously as the electropores resealed. The formation of electropores depends specifically on the cells that are used and the capacity and duration of the electric pulse that is applied to cells. Electric currents can lead to dramatic heating of the cells that can result in cell death. The effects of heating are minimized by using relatively high amplitude of electrical current, a short duration pulse or by using two very short duration pulses (Sukharev *et al* ., 1992)

Electroporation process is performed with electroporators, purpose-built appliances that create an electro-magnetic field in a cell solution. The suspension of cells is pipetted into a glass or plastic cuvette that has two aluminum electrodes on its sides. For bacterial electroporation, typically a suspension of around 50 microliters is used. Before the electroporation, the suspension of the bacterial cell is mixed with the plasmid to be transformed. The mixture is pipetted into the cuvette, the voltage and capacitance are set, and the cuvette is inserted into the electroporator. Immediately after electroporation, one milliliter of liquid medium is added to the bacteria in the cuvette or in an Eppendorf tube, and the tube is incubated at the bacterial optimum temperature for an hour or more to allow cells recovery and plasmid expression, followed by bacterial culture on agar plates.

Successful elecroporation depends greatly on the purity of the plasmid solution used, especially its salt content. Solutions with high salt concentrations might cause an electrical discharge (known as arcing), which often reduces the bacterial cell viability. For a further detailed investigation of the process, more attention should be paid to the output impedance of the porator device and the input impedance of the cells suspension (e.g. salt content). As the process needs a direct electrical contact between the electrodes and the suspension, and is inoperable with isolated electrodes, obviously the process involves certain electrolytic effects, due to small currents and not only fields.

Figure (1-2) display the external application of the electric field reacheing to the threshold values of the cell membrane, then cell membrane can be permeabilized to deliver protein, small and large molecules inside the cell and if two single cells are close to each other, then cell fusion can occur. To apply an intense electric field, which exceeds certain critical value, irreversible electroporation can occur resulting in to cell membrane rapture and finally cell death (Kanduser and Miklavcic, 2008).

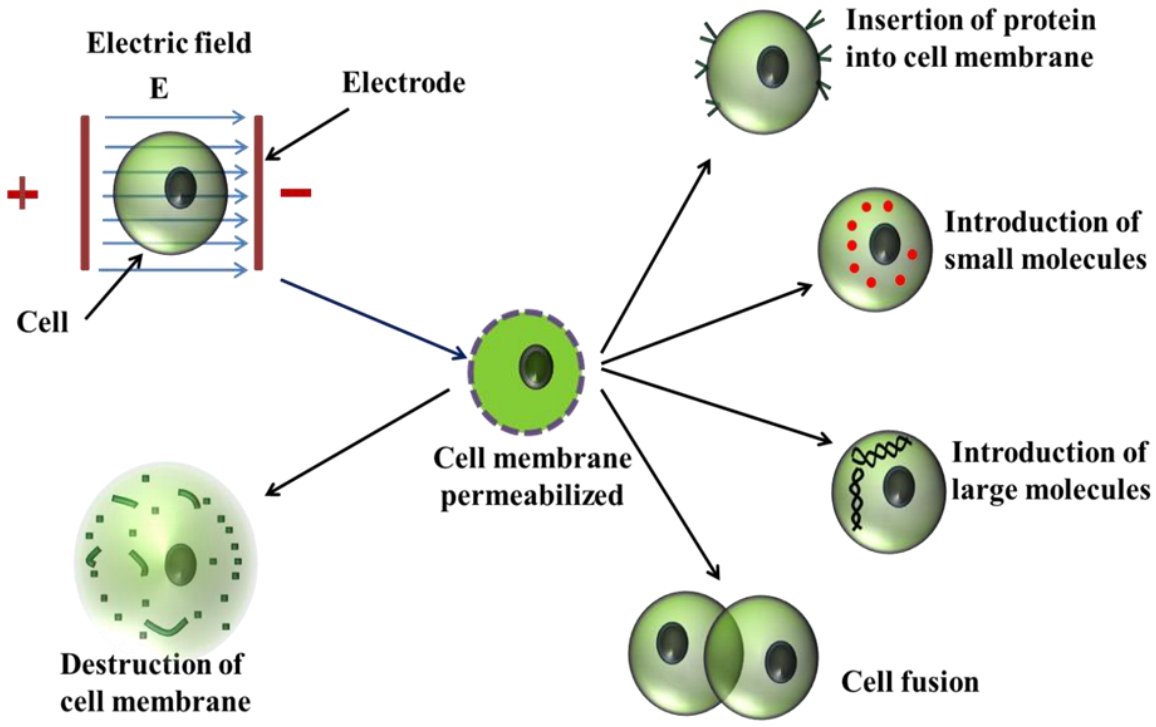


Figure (1-2): Different applications of single cell electroporation (Kanduser and Miklavcic, 2008).

1.2.8 Plasmids:

The term plasmid was first introduced by the American molecular biologist Joshua Lederberg in 1952 (Lederberg, 1952). A plasmid is a small DNA molecule that is physically separate from, and can copy itself independently of, chromosomal DNA within a cell. The plasmid mostly found as small circular, double-stranded DNA molecules in bacteria, sometimes present in archaea and eukaryotic organisms. In nature, plasmids carry genes that may provide survival of the organism (e.g. antibiotic resistance), and can be frequently transmitted from one bacterium to another even of another species via horizontal gene transfer. Artificial plasmids are widely used as vectors in molecular cloning projects, as to drive the replication of recombinant DNA molecule within host organisms (Lipps, 2008).

1.2.8.1 Specific Plasmid Functions:

Some studying projects will involve specific downstream applications that required specialized plasmid functions that are only present on some plasmids. For example, both the pBluescript and pUC series of vectors are high-copy-number, ampicillin-resistance- conferring plasmids that containing MCSs which facilitate the use of a wide range of restriction endonucleases in the cloning experiment step. However, one charecteristic present on pBluescript vectors that is not present on pUC vectors is promoters flanking the MCS that permit transcription of the insert DNA on either strand. The two promoters T7 and SP6 are recognized by bacteriophage RNA polymerases that must be supplied *in trans*. They do not transcribe host genes or other plasmid genes, enabling specific transcription of the insert DNA (Couturier *et al.*, 1998).

1.2.9 Cloning vector:

A DNA molecule that carries foreign DNA into a host cell, replicates inside a bacterial (or yeast) cell and produces many copies of its DNA content and the foreign DNA. The DNA cloned in these vectors are not always express themselves at transcription or translation level. These vectors mostly used for creating genomic projects or for preparing the probes or in genetic engineering studies or other basic programs. Cloning vectors Selection depends on the cloning experiment object, ease of working, knowledge existing about the vector, suitability and reliability. A variety of small, autonomously replicating molecules are used as cloning vectors (Voet *et al.*, 1999).

A vector is independently replicating DNA into which a foreign DNA is inserted for transfer or propagation in an organism. In general, a vector should have an autonomous specialized and designed to conduct a specific function. The most important criteria of vectors that make it useful for molecular cloning are listed below:

- Ability to self-replicate.
- Origin of replication.
- One and often multiple, restriction enzyme recognition site.
- Selectable characteristics so that the transformed cells may be recognized from untransformed cells.
- Promoter along with regulatory control.
- Less than 10 Kb in size.

1.2.9.1 Size of insert DNA:

For projects in which it is desired that a particular piece of DNA be cloned, one consideration is the size of the insert DNA. Most general cloning plasmids can carry a DNA insert up to around 15 kb in size. Inserts in excess of this place

constraints on proper replication of the plasmids (particularly for high-copy-number vectors) and can cause problems with insert stability.

Several types of vectors are available for cloning large fragments of DNA. These are most commonly used to construct libraries of clones that are often representative of entire genomes. Library clones are then screened to identify the particular clone that carries the DNA of interest (Losick. *et al*..2008)

1.2.9.2 Copy Number:

Different cloning vectors are maintained at different copy numbers, dependent on the replicon of the plasmid . In a majority of cases in which a piece of DNA is cloned for maintenance and amplification for subsequent manipulation, the greater the yield of recombinant plasmid from *E. coli* cultures, the better. In this scenario, a high-copy-number vector is desirable such as those whose replication is driven by the ColE1 replicon **(**Kahn *et al.*, 1979**).**

1.2.9.3 Incompatibility

Incompatibility refers to the fact that different plasmids are sometimes unable tocoexist in the same cell. This occurs if the two different plasmids share functions required for replication and/or partitioning into daughter cells. Direct competition for these functions often leads to loss of one of the plasmids from the cell during growth of a culture. Plasmid size can also influence maintenance within a culture, as larger plasmids require longer for replication and, thus, may be outcompeted by faster replicating of smaller plasmids (Del Solar and Espinosa, 2000).

1.2.9.4 Selectable Marker:

A selectable marker is carried by the vector to allow the selection of cells transformation . Resistance to antibiotic is often used as marker, an example is the beta-lactamase gene which grant resistance to the penicillin group of beta-

lactam antibiotics like ampicillin. Some cloning vectors contain two selectable markers, for example the plasmid pACYC177 has both kanamycin and ampicillin resistance gene (Casali and Preston 2003). Shuttle vector which is designed to be retained in two different organisms may also two selectable markers required , although some selectable markers such as resistance to hygromycin B and zeocin are effective in different cell types. Auxotrophic selection markers that allow an auxotrophic organism to grow growth medium with minimum nutrient requirement may also be used **(**Romanos et *al.*, 1992)

1.2.9.5 Cloning Sites:

All cloning vectors have an characteristics that allow a gene to be easily inserted into the vector or deleted from it .The DNA cloning into a vector usually involves ligation of the insert DNA fragment to vector DNA that has been cut with a specific restriction enzyme. This is enhanced when the insert and vector DNA fragments having compatible cohesive ends. Thus, the selected vector may be one that has a restriction endonuclease site that is appropriate with the insert fragment-generating enzyme. It should be noted, however, that any blunt-end fragment can be ligated to any other blunt-end fragment and that even DNA fragments generated by restriction enzymes that generate overhangs can be made blunt ended. In many older vectors, the restriction endonuclease sites were dispersed around the plasmid and were often in one of the vector genes.

More modern vectors often have an artificial extent of DNA that has a high concentration of restriction endonuclease sites that do not occur elsewhere on the plasmid. These multiple-cloning sites (MCSs) or polylinkers give a wide choice of restriction endonucleases for using in the cloning experiment step. They also determine the cloning site to one small region of the vector and also allow the specific positioning of the insert DNA close to other features of the vector (Esposito *et al.*, 2009).

1.2.10 Transformation in *Lactobacillus*:

Lactobacillus and many other lactic acid bacteria (LAB) are "generally regarded as safe" (GRAS) organisms and have the ability to efficiently secrete recombinant proteins directly into the culture medium. Thus, they are recognized as rising candidates for the expression of newly recombinant proteins as well as for genetic and metabolic cell engineering, both in the fields of medical and industrial biotechnology (Xu *et al.*, 2011**;** Nguyen *et al.*, 2011)**.** From the past century the interest in developing new and genetically engineered LAB strains with improved properties has been continuously growing, e.g. to produce cellulose degrading enzyme activity (Marcuschamer and Stephanopoulos**,**2007)

Lactic acid bacteria such as *Lactobacillus acidophilus, Lb. delbrueckii sbsp. bulgaricus, Lb. casei, Lb. helveticus, Lb. fermentum, Lb. plantarum and Lb. reuteri, and Bifidobacterium* species, are considered to be probiotics. Among these probiotics, lactobacilli including *L. acidophilus* play an important role in human health and nutrition by its effects on the intestinal microflora (Naidu et al. 1999) as well as in food fermentation industry. In view of the industrial and medical importance of lactobacilli, improvement of the characteristics of lactobacilli is one of major interests. For many years, enhanced strains have been selected by only classical techniques (Posno *et al.*, 1991). But these techniques are time consuming and ineffective for expression for specific and desirable characteristics. Genetic engineering techniques have been used to improve desired characteristics of LAB. Techniques such as transduction, conjugation, fusion and transformation have been used to transfer plasmid to produce genetically modified strains. Recently, transformation studies of lactobacilli have progressed in tandem with attempts to use them as a vehicle for heterologous protein expression including oral vaccines (Wells and Allison 1995; Pouwels *et al.*, 1998). Gene transfer systems for the strains of *Lactobacillus* using conjugation and protoplast transformation have shown low efficiency and lack of reproducibility (Vos and Simons 1994).

Electroporation is being used as one of the easiest methods to perform a genetic transformation. Since Harlander and McKay (1984) reported a successful application of electroporation to streptococci, a widespread interest in the possibility of bacterial electrotransformation had been prompted. *Lactobacillus casei* was the first lactobacilli to be transformed reproducibly by electroporation at high frequency and efficiency (Chassy and Flickinger 1987).

A number of parameters including the growth phase, cell density, medium compositions and electric conditions have been reported to affect the electrotransformation efficiency (Trevors *et al.*, 1992). Especially the electric conditions such as electrical pulse strength and length must be tested for the optimization of electroporation efficiency for a certain species of bacteria (Berthier *et al,.* 1996).

A DNA molecule that carries foreign DNA into a host cell, replicates inside a bacterial and produces many copies of its DNA content and the foreign DNA. The DNA cloned in these vectors are not always express themselves at transcription or translation level. These vectors mostly used for creating genomic projects or for preparing the probes or in genetic engineering studies or other basic programs . Cloning vectors Selection depends on the cloning experiment object, ease of working, knowledge existing about the vector, suitability and reliability. A variety of small, autonomously replicating molecules are used as cloning vectors (Voet *et al.*, 1999).

CHAPTER TWO

MATERIALS AND METHODS

2. Materials and Methods

2.1 Materials

2.1.1 Apparatus and equipment:

Apparatus	Company (Origin)
Anaerobic jar	Rodwell (England)
Autoclave	Gallenkamp (U.K.)
Balance (sensitive)	Ohans (France)
Compound light microscope	Olympus (Japan)
Cooling centrifuge	Harrier (U.K.)
Distillater	GFL (Germany)
Electrical incubator	Gallenkamp
Electrical oven	Gallenkamp
electroporator	Eppendorf/Germany
Gas generating kit	Rodwell
Gel documentation system	Bio-Rad (U.S.A.)
Gel electrophoresis system	Bio-Rad
Glass Pasteur pipettes	John Poulten Ltd. (England)
Hot plate with magnetic stirrer	Gallenkamp
Laminar air flow	Esco, Singapore
Microwave oven	Samaung (Korea)
Micropipette	Oxford (U.K.)
Nanodrop spectrophotometer	AE labs (U.K.)
pH-Meter	Meter-GmpH Tdedo (U.K.)
Refrigerated centrifuge	Harrier (U.K.)
Shaker incubator	Sanyo (Japan)
Spectrophotometer	Shimadzu, Japan
Thermal cycler	Applied Biosystem (USA)
UV transilluminator	AE labs
Vortex	Buchi (Sweeden)

Water bath	GFL

2.1.2 Biological and chemical materials:

Material	Company(Origin)
K_2HPO_4, KH_2PO_4 ,NaOH ,$CaCO_3$,KOH , NaNO3, Tris-HCl , MgCl2, $FeSO_4.7H_2O$, KCl , NaCl , HCl , MnCl2.4H2O , ZnSO4.7H2O , MgSO4.7H2O , CuSO4.5H2O Tiger milk , Glycerol , Asparagin , Casamino acid , Tyrosin , Sodiumdodecyl sulphate SDS, Sodium acetate , EDTA , Glacial acetic acid, Glucose, Mannitol , Acetic acid , Acetone , Ethanol.	BDH (UK)
Agar-Agar , Casein , Chlorophenol (Red) , Tryptone , Peptone , maltose , dextrose , Yeast extract, Malt extract , Soybean meal	Fluka (Switzerland)
Beef extract, Gelatin , Tween 80	Oxoid (UK)
Lysozyme , Ethidium bromide ,Agarose , Nuclease free D.D.W	Biolab (USA)
DNA ladder	Favor gene (USA)
Xylose, Arabinose, Sucrose, Raffinose	Difico (USA)
Isopropyl alcohol , Ninhydrin,potassium acetate	Sigma (USA)
Triton-X	Promega (USA)

2.1.3 Culture media

2.1.3.1 Ready-to-use powder media:

Medium	Company (Origin)
Mann, s Rogosa and Sharp (MRS) agar	Sigma (USA)
Mann, s Rogosa and Sharp (MRS) broth	Himedia (India)
Litmus milk broth	Biolife (Italy)
Blood agar base	Fluka (Switzerland)
Urea agar base	Fluka
Muller Hinton agar	Fluka
Nutrient agar	Sigma
Nutrient broth	Sigma
Simmon citrate agar	Difico (USA)
MacCkonky agar	Fluka
Urease broth	Fluka

2.1.3.2 Laboratory-prepared media:

ISP2(international streptomyces project) medium , Glycerol – asparagines agar, Melanin production medium , Czapeck-Dox medium, Sugars utilization medium, Sabaroud maltose agar, Asparagine- dextrose–meat extract agar, Bennet's Agar, Malt yeast agar, Starch agar, Gelatinase medium, Isp1, Isp 4, ISP5, Luria–Bertani (LB) medium, Hygromycine B antibiotic production medium, Blood agar

2.1.4 Solutions, Buffers, Reagents, and Dyes:

2.1.4.1 Ready-to-use:

Item	Company (origin)
Kovacs reagent	Chemistry Dept.(Al-Nahrain Uni.)
Catalase reagent	Al-Mansor Company (Iraq)

TBE buffer	Sigma (USA)
Loading dye	Promega(USA)

2.1.4.2 Laboratory-prepared:

-Ethedium bromide dye solution.

-Lysozyme reaction solution.

-Plasmid isolation solution.

-TBE buffer 1X.

-TLC mobile phase buffer.

-Tris- EDTA (TE) buffer.

-Buffer and solution (for bacterial transformation).

-Ninhydrin spray reagent.

2.1.4.3 Test bacteria: (Al–Nahrain Biotechnology Research Center, Iraq):

Saccharomyces cerevisiae, Klebseilla pneumoniae Salmonella typhimurium, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa and Staphylococcus aureus.

2.1.4.4 *Streptomyces* isolates: Local isolates obtained from (Al–Nahrain Biotechnology Research Center, Iraq):

Concatenation	*Streptomyces* symbol	Concatenation	*Streptomyces* symbol
1	2/G50 yellow	6	SH – 1
2	NS – 36	7	8D
3	LMS-25	8	LMS-5-9
4	35/10cm	9	SH-5
5	2/G50 gray	10	NS-12

Concatenation	*Streptomyces* symbol	Concatenation	*Streptomyces* symbol
11	11/G5	34	12/GS
12	7/30	35	Orange stain
13	Y2/34	36	Action surface 1cm
14	11/G5	37	10cm/Gs
15	1/G10 brown	38	10cm/36
16	AB-38	39	4/G50
17	ME-16	40	2/G50
18	SN10	41	2/G50*pigment
19	1/G10	42	1/G10
20	1/2/34	43	1/G10M
21	1/G10 pink	44	10CM/35+pigment
22	10cm/35	45	11/G.5
23	SN9	46	20/G10*
24	11/GS yellow	47	20/G10 16-spore
25	IQ	48	10cm/35 pigment
26	LMS-37-10	49	12/28
27	4/G50	50	12/34m
28	10cm/35 white	51	11/G.S
29	10cm/36	52	12/34m
30	1/2/34	53	10cm/35pigment
31	10cm/36**		
32	10cm/35ac		

33	7/30		

2.1.4.5 Plasmids:

pTKIP-hph	**Addgene (USA)**

2.1.4.6 Primers:

The following oligoprimers were designed to be used in this syudy (Shinegene / China)

Primer	**Sequence**	**Predicted size(bp)**
Hygromycin B gene primers		
Hygro-1-F Hygro-1-R	'5- TCGTATCCGCCAATGAGTCG -3' 5'- TTGCGTTCGGAGACGAAGAA -3'	454
Hygro-2-F- Hygro-1-R	'5- GATCGTGATCCTCTGCCAGG -3' '5- TGCAGTATCAGGACCCCGTA -3'	570
Hygro3-F- Hygro-1-R	'5- TGTGCGTCGGTCATCAAGAA-3' '5-GTAGATGGTGATCAGCCGGG-3'	866
Hygromycin B resistance gene primers		
HYGN-1-F PHYGN-1-R	5'-TTCAGCGACCAGTGCTCATT-3' 5'-GGGCAAGCAGGACCACTATC-3'	327
HYGN-2-F HYGN-2-R	5'-AGCGACCAGTGCTCATTGAC-3' 5'-GGCAAGCAGGACCACTATCT-3'	323

***Lacctobacillus* identification primer (16s rRNA)**		**Refrence**	**size(bp)**
IDL22R 2079 – 2104	5'AACTATCGCTTACGCTACCACTTTGC-3'	Kwon *et al.*,(2004)	6006bp

2.1.4.7: kits:

kit	**Company/origin**
Genomic DNA isolation kit	Favorgene / USA
Plasmid DNA isolation kit	Favorgene
PCR purification kit	Bioneer / Korea

Api 50CHl kit	Biomerieux,Marcy l' Etoile,/ France

The genomic DNA isolation kit was provided with the following buffers and lyses solutions:

Buffer	Solution
FATG2	Proteinase K
Washing buffer (W1)	Lysozyme
Washing buffer	
Elution buffer	

2.2 Methods:

2.2.1 Sampling

Milk fortified with probiotic was obtained from local market used as a sample for isolation of *Lactobacillus acidophilus*. The size of the container was 250ml.

2.2.2 Media Preparation:

2.2.2.1 Preparation of ready-to-use powdered media:

The media listed in (2.1.3.1) were prepared according to the information fixed on their containers by the manufacture. After pH was adjusted, the media were sterilized in the autoclave unless otherwise stated.

2.2.2.2 Preparation of laboratory-prepared media:

a) Yest xtract-Malt extract agar **ISP2** : (Shirling and Gottlieb, 1966.)

All ingredients were dissolved in 950ml of distilled water , pH was adjusted to 7 , then volume was completed to 1000ml with distilled water and sterilized by autoclaving.

Ingredient	**weight(g)**
Yeast extract	4
Malt extract	10

Dextrose	4
Dipotassium phosphate	0.1
Tryptone	2
Agar	15

b) Luria – Bertani (LB) medium with hygromycine B :

It was prepared according to Gerhardt *et al.* (1994) from:

Ingredient	**weight(g)**
Tryptone	1
Yeast extract	5
NaCl	10
Hygromycine B	100µg/ml
D.W	1000ml

After the first three ingredients were dissolved in water, and autoclaved, hygromycin B previously sterilized by filtration, was added.

c) Czapeck-Dox medium: (Thom and Church, 1926)

All ingredients were dissolved in 950ml of distilled water , pH was adjusted to 7 , then volume was completed to 1000ml with distilled water and sterilized by autoclaving.

Ingredient	**weight(g)**
Sucrose	30
Sodium nitrate	2
Dipotassium phosphate	1
Magnesium sulfate	0.5
Potassium chloride	0.5
Ferrous sulfate	0.01

Agar	15

d) Glycerol–asparagines agar ISP5 (Pigment formation medium):

It was prepared according to Shirling and Gottlieb (1966) All ingredients were dissolved in 950ml of distilled water , pH was adjusted to 7 , then volume was completed to 1000ml with distilled water and sterilized by autoclaving.

Ingredient	**weight(g)**
Glycerol	10
Asaparagine	0.5
K_2HPO_4	0.5
$FeSO_4. 7H_2O$	0.001
$MnCl_2. 4H_2O$	0.001
$ZnSO_4. 7H_2O$	0.001
Agar	20

e) Melanin production medium: (Hopwood *et al.*, 1985)

All ingredients were dissolved in 950ml of distilled water , then 1.5ml of tiger milk ,10ml of tyrosine (0.75%) and 4ml of casamino acids(30%) were added, volume was completed to 1000ml with distilled water , pH was adjusted to 7 and sterilized by autoclaving.

Ingredient	**weight(g)**
L – asparagines	0.5 g
K_2HPO_4	0.5 g
$MgSO_4. 7H_2O$	0.2 g
Glucose	10 g
Agar	10 g

Solutions : Tiger milk , Casamino acids (30 %) and L – tyrosine (0.75 %)

f) Sugars utilization medium: (MacFaddin, 2000)

This medium was prepared by dissolving the first four ingredient in D.W. and autoclaved then 1% of membrane filtered sugar was added.

Ingredient	**(g/l)**
peptone	10 g
Sodium chloride	5 g
Beef extract	1 g
Phenol red	0.018
Deionized-Distilled water	1,000 ml
Carbohydrate (D-glucose, Inositol, L-arabinose, Sucrose, D-xylose, ructose, Mannitol	10 g
D.W.	1000ml

g) Sabouraud maltose agar: (Chapman ,1952 **)**

All ingredients were dissolved in 950ml of distilled water , pH was adjusted to 5.6 , then volume was completed to 1000ml with distilled water and sterilized by autoclaving.

Ingredient	**weight(g)**
Mycological peptone	10
maltose	40
agar	15

h) Asparagin – dextrose – meat extract agar

It was prepared according to Deshpande *et al.*, (2014), all ingredients were dissolved in 950ml of distilled water , pH was adjusted to 7 , then volume was completed to 1000ml with distilled water and sterilized by autoclaving.

Ingredient	**weight(g)**

Glucose	10
asparagine	0.5
K2HPO4	0.5
Meat exract	2
agar	17

I) Bennetts agar

This culture medium was prepared according to Murray, *et al.*, 2003, all ingredients were dissolved in 950ml of distilled water , pH was adjusted to 7.3 , then volume was completed to 1000ml with distilled water and sterilized by autoclaving.

Ingredient	**weight(g)**
Beef extract	1
Glucose	10
n-z aminoA(enzymatic digest of casein)	2
Yeast extract	1
agar	15

Malt-Yeast agar (Trensner *et al* ., 1960)

All ingredients were dissolved in 950ml of distilled water , pH was adjusted to 7, then volume was completed to 1000ml with distilled water and sterilized by autoclaving.

Ingredient	**weight(g)**
yeast extract	4
Malt extract	10
glucose	4

Agar	20

K) Starch agar:

It was prepared according to Atlas *et al*., (1996) All ingredients were dissolved in 950ml of distilled water , pH was adjusted to 7, then volume was completed to 1000ml with distilled water and sterilized by autoclaving.

Ingredient	**weight(g)**
Beef extract	3g
starch	10g
agar	15g

k) Gelatin medium: (Atlas *et al*., 1996).

All ingredients were dissolved in 950ml of distilled water , pH was adjusted to 7 , then volume was completed to 1000ml with distilled water, dispense 2-3 mL of media into culture tubes and sterilized by autoclaving.

Ingredient	**weight(g)**
Peptone	5.0 g
Beef extract	3.0 g
Gelatin	120.0 g

l) Tryptone-yeast extract agar (Isp 1):

It was prepared according to Pridham and Gottlieb (1948) , all ingredients were dissolved in 950ml of distilled water , pH was adjusted to 7, then volume was completed to 1000ml with distilled water and sterilized by autoclaving

Ingredient	**weight(g)**
Pancreatic Digest of Casein	5
Yeast Extract	3

agar	20

m) Inorganic salt- starch agar (Isp 4) :

It was prepared according to Shirling and Gottlieb (1966.), all ingredients were dissolved in 950ml of distilled water , pH was adjusted to 7, then volume was completed to 1000ml with distilled water and sterilized by autoclaving

Ingredient	**weight(g)**
Soluble Starch	10
Dipotassium Phosphate	1.0
Magnesium Sulfate	1.0
Sodium Chloride	1.0
Ammonium Sulfate	2.0
Calcium Carbonate	2.0
Ferrous Sulfate	0.001
Manganous Chloride..	0.001
Zinc Sulfate	0.001
Agar	20

n) Hygromycin B production medium: (Poosarla *et al*., 2013)

All ingredients were dissolved in 950ml of distilled water , pH was adjusted to 7, then volume was completed to 1000ml with distilled water and sterilized by autoclaving

component	**weight(g)**
Soyabean meal	10
glucose	10
NaCl	10
CaCO3	1

o) Blood agar;

This medium was prepared by autoclaving blood base agar and cooling it to 45˚C, then 5% of sterilized blood was added to it before mixing gently and poured into Petri dishes.

2.2. 3 Preparation of Solutions, Buffers, Reagents and Dyes:

- **Lysozyme reaction solution: It was prepared from 4 components:-**
  - a- Lysozyme (20 mg/ml).
  - b- Tris-HCI, pH 8.0 (20 mM).
  - c- EDTA (2mM).
  - d- Triton X (1.2 %).
- **Plasmid isolation solution: It was prepared from 4 components:-**

a- Resuspension buffer: composed from; Tris-HCl, pH 8 (25 mM), Glucose (50 mM), and EDTA (10 mM). pH was adjusted to 8 before storing at 4°C.

b- Denaturizing solution: composed from; NaOH (0.2 N) and SDS (1%), it was stored at room temperature.

c- Renaturing Solution: Composed of; Potassium acetate, 5M (120 ml), Glacial acetic acid (23 ml) and D.W. 57 ml. Solution was stored at 4°C.

d- Sodium-acetate, 3M: prepared by dissolving 1.23g from the material in 5 ml D.W., then pH was adjusted to 4.8 before storing at room temperature.

- **TBE buffer 1X: It was** prepared by adding 100 ml of TBE buffer stock solution (10X) to 900 ml D.W.
- **TLC mobile phase buffer: (Pauncz , 1972)**

This buffer was consisted of 1.5 M sodium acetate solution, 1.0 M sodium chloride and 10 % butanol, to give a pH of 8.5.

- **Tris- EDTA (TE) buffer: (Maniatis *et al.*, 1982)**

It was consisted of 10 mM Tris-HCl, and 0.1 mM EDTA with a pH of 8.

- **Ninhydrin spray reagent :**

This reagent was prepared by dissolving 1g of ninhydrin in 100 ml of n-butanol, then 3ml of acetic acid and 3ml acetone were added. The reagent was mixed by the magnetic stirrers for dissolving the ninhydrin.

2.2.4 Sterilization methods :

2.2.4.1 Moist sterilization (Autoclaving):

Media and solutions were sterilized by the autoclave at 121˚C (15Ib/inch^2) for 15 minutes .

2.2.4.2 Membrane Sterilization (Filtration):

Membrane filter with pores of (0.22) µm were used to sterilize some sugar solutions.

2.2.5 Maintenance of isolates:

2.2.5.1 Working culture:

Maintenance of bacterial isolates was performed by using sterile ISP4 broth which inoculated with bacterial isolates and incubated at 30˚C for 5 days, then kept in the refrigerator and reactivated weekly.

2.2.5.2 Stock Culture :

The isolates were grown on agar slants of malt-yeast agar in screw-caped test tubes at 28 C for 14 days, after which they were kept at -18^{o}C. In a deep freeze .

2.2.6 Isolate collection:

From Al – Nahrain Biotechnology Research Center, 53 *Streptomyces* isolate was taken, that identified according to their morphological , cultural , and biochemical test . DNA was extracted from all these isolates for screening of hygromycin B gene.

2.2.7 DNA extraction protocol:

For the isolatation and purification of DNA from S*treptomyces* isolates, DNA extraction Kit was used according to the manufacturing company (favor gene, USA) protocol and recommendations as follow:

- One ml of well-grown bacterial culture was transferred to a microcentrifuge tube.
-The bacterial cells were centrifugated at full speed (15000rpm) for 2 min, then the supernatant was discarded and the pellets obtained.
- The cell pellets were resuspended in 200 µl lysozyme-reaction solution before incubattion at 37°C for 30 min.
- A portion of 20 µl Proteinase K and 200 µl FATG2 Buffer were added to the sample, and then mixed thoroughly by the pulse-vortexing.
- The suspended cells were incubated in the water bath at 60 °C for 30 min and then for a further 15 min at 95 °C.
- The tube was spin shortly to remove drops from the inside of the lid.
- A portion of 200 µl ethanol (96-100%) was added to the sample, and mixed thoroughly by the pulse-vortexing.
- The tube was spin shortly to remove drops from the inside of the lid.
- FATG Mini Column was placed in the Collection Tube, the mixture was transfered (including any precipitate) carefully to FATG Mini Column and Centrifuged for 1 min, then placed FATG Mini Column to a new Collection Tube.
– The FATG Mini Column and 500 µl Wash Buffer were centrifuged for 1 min then the flow-through was discarded.
- the FATG Mini Column with another 750 µl Wash Buffer were centrifuged for 1 min then the flow-through was discarded. (Note: the ethanol must be added into the Wash Buffer when first open).
- The column was centrifuged for an additional 3 min to dry completely.
- The FATG Mini Column was placed into the Elution Tube.
- A portion of 200 µl of Elution Buffer (pH 7.5-9.0) was added to the membrane center of FATG Mini Column before standing the FATG Mini Column for 3 min.

(Note: the elution solution should be dispensed onto the membrane center and absorbed completely.

- The FATG Mini Column was centrifuged for 2 min to elute the total DNA. - The DNA was stored at 4°C or -18°C.

2.2.8 Quantization of DNA concentration:

Concentration and purity of DNA samples were measured according to (Maniatis *et al.*,1982) by using the computerized nanodrope– spectrophotometer where 2µl of DNA sample were added into the photocell of the apparatus. The results of DNA concentration and purity were recorded and plotted in the device of the apparatus automatically.

Nanodrop spectrophotometer result calculations depend on the following equation

$$\textbf{DNA purity} = \frac{Abs.at\ 260nm}{Abs.at\ 280nm}$$

DNA concentration (ng/µl)= Abs (260nm) x 50µg/ml

Note: Absorbency (260nm) of 1 equal to 50µg/ml of pure DNA.

2.2.9 Hygromycin B gene

For all DNA samples, Hygromycin B gene were detected using specific desigend PCR primers as follows:

2.2.9.1 Amplification of Hygromycin B gene using PCR:

To amplify the gene for Hygromycin B production from *Streptomyces hygroscopicus*, basic information to design specific primers was obtained from database of DNA sequences in NCBI . Primers used to amplify the gene listed in 2.1.4.6

The Primers were completely designed to base pair with the genetic element sequences of the gene.

The reaction mixture (25 µl) for Hygromycin B gene amplification by PCR contained the following components:

component	Final Conc.(pmol)	Volume (µl)
DNA sample	10pmol	10µl
Primers	1pmol	1 µl
Master mix	1X	14 µl

Amplification procedure was performed according the following program after optimization experiment:

primers / Step	Hygro1			Hygro2			Hygro3		
	Temp. (°C)	Time (min)	No. of cycles	Temp. (°C)	Time (min)	No. of cycles	Temp (°C)	Time (min)	No. of cycles
Initial denaturation	94	5	1	94	5	1	94	5	1
Denaturation	95	2	35	95	2	35	95	2	35
Annealing	64	30Sec	35	60	30Sec.	35	58	30Sec	35
Extension	72	1	35	72	45sec.	35	72	45Sec.	35
Final extension	72	7	1	72	7	1	72	7	1

2.2.9.2 Gel Electrophoresis of PCR products:

The products of PCR were analyzed on agarose gel (1.5%) by using horizontal electrophoresis unit, PCR products loaded into the wells , then gel was immersed in 1X TBE buffer. Electrophoresis was carried out for 1.5 h at 5v/cm. After that, gel was stained with ethedium bromide solution for 20 min, then DNA bands were visualized by using UV transilluminator.

2.2.10 Detection of antimicrobial activity:

Antimicrobial activity for only the *Streptomyces* isolates that gave positive results in Hygromycin B gene detection was performed after the broth bacterial culture was centerfugated and using agar well diffusion method against the six

pathogenic isolates (2.1.4.3) and then the most active isolate was selected for further identification and study.

2.2.11 Identification of *Streptomyces* isolates:

Streptomyces isolate gives the highest antimicrobial activity was subjected to the microscopic, cultural, and biochemical examinations for their identification as follow:

2.2.11.1 Microscopic examination: (Riddell, 1950)

Microscopic examination was performed by using slide culture technique, where a loopfull of bacterial culture was streaked on ISP4 plate, and then aseptically, a sterile cover glass was placed on the upper surface of the agar. After incubation for 7 days at 30^{o}C, the slide was picked up and examined under light microscope.

2.2.11.2 Cultural examinations: (Shirling and Gottlieb 1966.)

Cultural examinations of the suspected *Streptomyces* isolates included; substrate mycelium, nature and formation of aerial mycelium, structure and branching of pseudohyphae containing spores, and spore surface. Growth on different media, color of aerial and substrate mycelia, and formation of soluble pigments were also performed.

A loopfull from the culture of *Streptomyces* isolate was streaked on each of the following media and incubated at 30^{o}C for 5 days.

- ISP2
- ISP5(pigment formation medium)
- Melanin production medium.
- Czapeck Dox agar medium
- Sabaroud maltose agar

- Asparagine–dextrose–meat extract agar
- Bennetts agar
- Isp 1
- Isp 4
- ISP 3
- PDA
- Nutrient agar

2.2.11.3 Biochemical tests:

- Catalase Test:

This test was performed by adding 2-3 drops of hydrogen peroxide (3%) to the mass of bacterial cells placed on the microscopic slide. Production of gaseous bubbles indicates a positive result (Atlas *et al.*, 1995).

- Gelatinase Test :

Gelatine liquefaction was detected by inoculating gelatin medium with 1% of bacterial culture and incubated at 30˚C for 5 days. After that, it was put in the a refrigerator (4˚C) for 30 minutes. Positive result was observed by gelatin liquefaction (Baron and Fingold , 1994).

-Indole Test:

Tryptone broth was inoculated with 1% of the bacterial isolate and incubated at 37˚C for 5 days. After that, (0.5) ml of Kovacs reagent was added and shaked . Positive result was recorded by appearance of red layer at the top of the broth (Atlas *et al.*, 1995).

- Starch Hydrolysis Test:

Starch agar was inoculated by a loopfull of bacterial isolate and incubated at 30˚C for 5 days, then the plate was flooded by iodine solution. Positive result was

indicated by the formation of clear zone around the colonies while the medium stained by blue color (Atlas *et al*., 1995).

- Carbohydrate Fermentation test:

Carbohydrate fermentation medium was inoculated with 1% of bacterial culture and incubated at 30˚C for 5 days. Color changing from red to yellow indicates positive result (Atlas *et al*.,1995) .

- Blood hemolysis test:

Blood agar medium was inoculated by a loopfull of bacterial isolate and incubated at 30˚C for 5 days, then formation of hemolysis was observed as a positive result.

- citrate utilization test:

A tube of Simon citrate agar was inoculated by a loopfull of bacterial isolate and incubated at 30˚C for 5 days. After incubation, color changing from green to blue indicates positive result.

2.2.12 Amplification of Hygromycin B phosphtrasferase resistance (*hph*) gene using PCR:

To amplify the gene of Hygromycin B phosphtrasferase resistance (*hph*) from *Streptomyces hygroscopicus*, also basic information to design specific primers was obtained from database of DNA sequences in NCBI . Primers used to amplify the gene listed in 2.1.4.6 .

The reaction mixture (25 µl) for ***hph*** gene amplification by PCR contained the following components:

component	Final Conc.(pmol)	Volume (µl)
DNA sample	10pmol	10µl
Primers	1pmol	1 µl
Master mix	1X	14 µl

Amplification procedure was performed according the following program after optimization experiment for the two primers:

Step	Temp. (°C)	Time (min)	No. of cycles
Initial denaturation	94	2	1
Denaturation	95	45Sec.	35
Annealing	55	45Sec.	35
Extension	72	1	35
Final extension	72	7	1

The products of PCR were analyzed as in 2.2.9.2

2.2.13 Extraction of bioactive compounds:

Antibiotic production medium was inoculated with 1% of the selected *Streptomyces* isolate and incubated at 35°C for 7 days. After that filtration was carried out through No.1Whatman filter paper. The filtrate was centrifuged at5000 rpm for 15 min and antimicrobial activity was tested .

After pH was adjusted to 8 , the procedure proceed by extraction with ethyl acetate 1:1(v/v) in the rotary evaporator. The extract was separated into organic phase and aqueous phase; the organic phase was evaporated at room temperature for 4 days, while the aqueous phase evaporated in the oven at 40°C for 6 days. After the residual material was diluted with 5 ml of sterile distilled water, 1 ml was taken from each phase and put in a beaker previously weighted then dried and weighted again to calculate the concentration of the active compounds in 1ml of D.W. each part was tested for the antimicrobial activity by using agar diffusion method. The extract of the phase that gave biological activity was used for further Hygromycin B detection .

2.2.14 Detecting Hygromycin B by Thin Layer Chromatography (TLC) technique:

Thin Layer Chromatography (TLC) technique (Judit, 1972) was used for separation of the crude extract into its active compounds. A small spot from each of the aqueous extract and the Hygromycin B standard solution was applied to an aluminum foil coated thin-layer chromatoplates (20x20 cm) that placed in a separation chamber containing sodium acetate solution (eluent) as a mobile phase (taking in the consideration that the spots of the sample shouldn't touch surface of the eluent in the chamber). After the chamber was closed, it was left at 40°C **.** The solvent was then moved up via a capillary action and the sample was eluted, and the run was ended before the solvent reached the end of the plate. The spots was visualized by the ninhydrin spray reagent and heaing at 110 until the reddish spots appear and R_f value was calculated.

$$R_f \text{ Value} = \frac{\text{Distance from Baseline travelled by Solute}}{\text{Distance from Baseline travelled by Solvent (Solvent Front)}}$$

2.2.15 Optimization of Hygromycin B production conditions:

2.2.15.1 Growing at different pH:

Each of the six conical flasks, contained 25ml of hygromycin B production medium but with different pH values (4, 5, 6, 7, 8 and 9). They were prepared and inoculated with 1% of the exponentially growing culture of *Streptomyces* isolate, and incubated at 30˚C for 7 days. By using the agar diffusion method, result was determined by exerting antimicrobial activity against *S. aureus, Proteus mirabilis,* and *Saccharomyces cereviciae* through measuring diameters (mm) of inhibition zones.

2.2.15.2 Growing at different temperatures:

Each of the five conical flasks, contained 25ml of hygromycin B production medium with an optimum pH determined from the previous step prepared and inoculated with 1% exponentially growing culture of *Streptomyces* isolates then incubated at different temperatures (25 , 30 , 35, 40 , 45˚C) under aerobic condition for 7 days, By using the agar diffusion method, result was determined by exerting antimicrobial activity against

S. aureus, Proteus mirabilis, and *Saccharomyces cereviciae* through measuring diameters (mm) of inhibition zones.

2.2.15.3 Growing for different incubation periods:

In each of the five conical flasks, a volume of 25 ml of Hygromycin B production mediium with the optimum pH was inoculated with 1% of the active culture of *Streptomyces* isolate before incubation at the optimum temperature for different incubation periods (5 , 6 ,7 , 8,10 days). By using agar diffusion method, results were read through measuring the diameters (mm) of inhibition zones formed the inhibitory activity of Hygromycin B against the three test microorganisms.

2.2.16 Detection of Hygromycin B toxicity:

2.2.16.1 Dose preparation:

The dose prepared from the crude extract of Hygromycin B was in a concentration of (0.0002/gm body weight of mice, if we know ,practically, the recommended amount of Hygromycin B used as feed additive for chicken is 12g/ton and mean of chicken weight is 1500gm while the mean of mice weight is 15gm.

2.2.16.2 Treatment of mice:

Six adult mice, with an average weight of 15 gm / each, were obtained from Biotechnology Research Center at Al-Nahrain University. They were put in the animal house of the center. The mice were divided into two groups; the first was daily treated by orally administration of 100µl of Hygromycin B for 14 day, while the second group was left without treatment as a control.

2.2.16.3 Histological analysis of experimental mice:

After liver sample was kept in 10% formalin for 72 h, it was washed with water and kept in it for 2 h to discard the remaining formalin. Then, the sample was saved for 2 h in ascending concentrations of ethanol (70, 80, 90, 100%) to dehydrate the tissue, followed by 3 h in toluene to clear the sample from the alcohol. The sample was then incubated in paraffin wax embedding medium at 65°C for 4 h to remove the remaining toluene, followed by embedding in paraffin plastic mold until solidification and sectioning by microtome; the sections were fixed on glass slide and put in the oven at 57°C for tissue staining.

2.2.16.4 Staining procedure:

Staining procedure was began by passing the glass slide in descending concentrations of ethanol (90, 80, 70%) for 10 min, and then in distilled water for 5 min before stained by heamatoxylin for (7-10) min. After that, the slide was first immersed in distilled water for four times, then in acid alcohol for two times (or until the appearance of light red color) before transferring to the tap water for five min. Eosin stain was added for 5 minutes and then the slide was immersed in tap water 5-7 times. The following ethanol ascending concentrations and contacting times (70, 80% /2 min, 90%/5 min, 100% /10 min) were used for two times, then xylol was added for 10 min for two times also to dry the alcohol. Finally, the slide was cleaned and preserve by adding one drop of Canada balsam and covered with a cover slip to be examined by the compound light microscope.

2.2.17 Isolation of *Lactobacillus* spp

Serial dilutions from the yoghurt samples were prepared, 1ml from the last dilution was transferred , poured on MRS plates and incubated for overnight at 37˚C.

2.2.18 Identification of *Lactobacillus acidophilus* isolates:

2.2.18.1 Microscopic examination:

A loopful from the suspected colonies was fixed on a microscopic slide, then stained by Gram stain to examine Gram reaction, cells shape and grouping, and non-sporeforming (Kandler and Weiss , 1986 ; Garvie and Weiss ,1986).

2.2.18.2 API 50 identification of *Lb.* acidophilus isolates:

Identification of *Lactobacillus acidophilus* isolates was performed by using API 50 CHL, a standardized system which consisting of 50 biochemical tests for the study of sugar fermentation by microorganisms. API 50 CHL is used in conjunction with API 50 CHL medium for the identification of *Lactobacillus* and related genera strips according to the manufacturer's instructions (Biomerieux, Marcy l' Etoile, France, Ghanbari *et al,* 2009). A portion of 10 ml of D.W. was dispensed into the incubation box which contained the strip. Then, 5 ml of the bacterial cultures had been inoculated into the API 50 CHL system medium (in concentration of 2 McFarland). The set-up system was then incubated at appropriate temperature of (35°C) for 48 h, then the wells were filled with the bacterial suspension with the addition of mineral oil. Bacterial isolates were identified based on their sugars fermentation. Identification tables were prepared as (+/-) according to color change in evaluation of results of API strips reaction. Numerical profiles of isolates were identified by adding positive values in indicative table.

2.2.19 DNA extraction of *Lb. acidophilus*:

DNA was extracted from *Lb.acidophilus* as the method described in item(2.2.7) .

2.2.19.1 Molecular identification of *Lb. acidophilus* isolates:

According to Kwon *et al.*,(2004) *Lactobacillus acidophilus* was identified by using specific and conserved primer(IDL22R 2079–2104) with a predicted product size of (606bp) based on the 16S and 23S rRNA genes and their intergenic spacer region(ISR) . DNA extracted in the above item was amplified using this primer and analyzed on agarose gel electrophoresis.

The reaction mixture (25 µl) amplification by PCR contained the following components:

component	Final Conc.(pmol)	Volume (µl)
DNA sample	10pmol	10µl
Primers	1pmol	1 µl
Master mix	1X	14 µl

Amplification procedure was performed according the following program:

Step	Temp. (°C)	Time (min)	No. of cycles
Initial denaturation	94	2	1
Denaturation	95	45Sec.	35
Annealing	55	45Sec.	35
Extension	72	1	35
Final extension	72	7	1

2.2.20 Plasmid extraction and purification protocol: (addgene protocol /USA)

Escherichia coli bacteria provided from (addgene / USA)containing the plasmid (pTKIP-HPH) was grown in Lb (Luria–Bertani medium) with 100 µg/ml Hygromicin B (as selective agent) for overnight at 37°C, then the plasmid was extracted according the following procedure:

- Into the 1.75ml microfuge tube, 1.5 ml of the stock culture was added before centrifuged at 10,000g for 30 seconds.

-The supernatant was poured off carefully to maintain the bacterial pellets from dispersing.

-The pellets were suspended in 100 µl of cold Solution I.

-The solution was vortexes for 2 min (or until all bacteria were fully resuspended).

-A portion of 200 µl of solution II was added and the tube was inverted carefully for 5 times to mix the contents. The contents should become clear and thicker as the proteins and DNA are denatured.

-The solution was incubated on the ice for 5 minutes.

-To each tube, 150 µl of cold Solution III was added.

-Tube contents were mixed by inverting several times. Formation of white precipitate indicates that the sample contains the bacterial proteins and genomic DNA.

-The tube was incubated on the ice for 5 minutes, before centrifugation for 5 minutes at 12,000g.

Note: *Pellet contains proteins, cell fragments, salt and other extra particles from solutions.*

Note: *Supernatant contains the plasmid DNA separated from bacterial chromosomes.*

-The supernatant was collected into a new tube by pipetting or carefully pouring and centrifugated for 5 minutes at 12,000g.

-The supernatant was collected into a new tube by pipetting or carefully pouring.

-Portions of 2-2.5 of 95% -100% ethanol and 1/10 volume of 3 M Na-acetate (pH 4.8) wree added to the DNA solution.

-The microfuge tubes were inverted for mixing before placed at -20°C for overnight (Note: Freezing may help precipitating the DNA).

-The solution was centrifuged in the cooling centrifuge (4°C) at 12,000 rpm for 15min.

Note: Pellets contain the precipitated DNA, while the supernatant (which was discarded) contains the residues, salts, and water

- The tubes were opened and inverted on a paper towel to drain them out.

-The pellets were washed by adding 500 µl of cold 70% ethanol (This helps to remove excess salt from the DNA pellets).

- The solution was centrifuge at 12,000 rpm) for 5 minutes at room temp., and the supernatant was discarded.

> Note: The pellets were harder to see and less well-attached to the tube after washing with the 70% ethanol. To avoid losing pellets, the supernatant can be pipette out.

-The pellets were dried for 5-20 min in the vacuum while the paper towel was inverted.

- DNA was resuspended in the TE buffer.

-The DNA was stored at 4°C.

2.2.21 Transformation of *Lb. acidophilus* by electroporation:

- Cells were precipitated by centrifuge (4 °C),and wash twice with 10 mL chilled MgCl2 (10 mM) and once with 10 mL of a chilled solution of sucrose (0.5 M) and glycerol (10 % w/v).
- The pellet was resuspend in 2-3 mL of the same solution and store until use in an ice bath, but no longer than 4 h.

Electroporation of cells:

- A pportion of 100 ng plasmid DNA was diluted in 5 µL TE buffer and to 50 µL ($3x10^8$) freshly prepared competent cells were added.Homogenize by gently mixing with pipette several times and transferring the mixture into a pre-chilled cuvette.

- Moisture was Wiping from the cuvette and then insert into the device.
- Electroporation was performed at Voltage (V) 1,300 V and time constant (t) 5 ms
- Immediately 1 mL of pre-warmed MRS medium was added and incubate at 30 ºC for 3 h with shaking.
- Plate on selective MRS agar plates and incubate al 30 ºC for 48 h.
- Transformation efficiency was calculated by making a serial dilution of transformed cell and growing on MRS plates containing 100µg/ml hygromycin B antibiotic and select the countable one.

CHAPTER THREE

RESULTS AND DISCUSSION

3. Results and Discussion

3.1 Genomic DNA extraction

William *et al.* (1997) stated that ˜1.8 is the ideal value for DNA purity when absorbed at 260 to 280 nm wavelength.

Results of extraction in table (3-1) showed that the 53 *Streptomyces* isolates were different in their values of DNA purity. The isolates that gave values within the range of (0.56 to below 1.8) reflecting the presence of protein molecules, phenol and any other contaminants in the samples because such contaminants are also absorbed at 280 nm wavelength. Some other isolates recorded values of purity higher than 1.8 which can be referred to the RNA residue may be present in the samples. On the other hand, two isolates (35/10cm and10cm/35) gave acceptable values of purity when these values were closed to the ideal one (1.8). The results of *Glasel (1995) showed that* The quality of the extracted DNA was evaluated by the A260/280 ratio, and values close to 1.8 indicate a good DNA extract with little protein contamination. Yang *et al*.(2008) also revealed the same results.

Table (3-1) Purity and concentration of DNA extracted from *Streptomyces* isolates.

concatenation	Isolate symbol	DNA Purity	DNA Concentration (ng)
1	2/G50 yellow pigment	2.11	١٠,٥
٢	NS - 36	1.78	78.90
٣	LMS-25	٢,٥١	٢٥,٨١
٤	35/10cm	١,٧٣	٢٣٠,٧٣
٥	2/G50 gray	١,٥	44.50
٦	SH – 1	١,٢٤	٢٤٨,٩٥
٧	8D	٢,٢	٢٩٣,٥٦
٨	LMS-5-9	٢,٠١	٤٢,٨٧
٩	SH-5	١,٦٩	١٢٨,٨١
١٠	NS-12	٢,٥٩	٢٣,٧٣
١١	11/G5	١,٩٣	٦٠٢,١٣
١٢	7/30	٢,٢٥	١٠٢,٠٦
١٣	Y2/34	٢,٣١	٢٣,٧٣

١٤	11/G5	١,٩٠	٤٠,٤٣
١٥	1/G10brown	٢,٣١	٣٧,٩٨
١٦	AB-38	١,٨٠	٢١٤,٧١
١٧	ME-16	٢,٥٥	٣٤,٣٣
١٨	SN10	٢,٢٠	٣٣,٧١
١٩	1/G10	١,٦٦	١٧,٩٨
٢٠	1/2/34	٣,١٩	٥٤,٢٥
٢١	1/G10 pink	١,٤٥	٤,٦٣
٢٢	10cm/35	١,٨٦	١٢,٧١
٢٣	SN9	٢,٣٨	٣١,١٥
٢٤	11/GS yellow	٢,٢	٩١,٥
٢٥	IQ	٢,١٥	١١,٨
٢٦	LMS-37-10	٢,٨٣	٥٦,٠٢
٢٧	4/G50	١,٠٣	٥,٢
٢٨	10cm/35 white	١,8	٩٠,٨٢
٢٩	10cm/36	٢,٥٢	٢١,٦١
٣٠	6mt	١,٩٧	٣٢,٤٤
٣١	10cm/36A	٠,٨٣	٢٤,٢١
٣٢	10cm/35ac	٢,١٣	٣٧,٣٦
٣٣	7/30	١,٧٨	٩٩,٦٣
٣٤	12/GS	١,٦	١٥,٧٥
٣٥	Orange stain	٠,٩٨	٥٠,٤٨
٣٦	Action surface 1cm	١,٩٦	٣١,١٥
٣٧	10cm/Gs	١,٣	٢٧٧,٥٤
٣٨	10cm/36B	١,٢٧	٢٦٣,٨٦
٣٩	4/G50	١,٢١	٢٣٧,٢١
٤٠	2/G50	٠,٨٤	٩٩,١٩
٤١	2/G50 pigment	1.6	120.32
٤٢	1/G10	١,١٥	١٧٥,١١
٤٣	1/G10M	٠,٨٣	٩٨,٥٧
٤٤	10CM/35+pigment	٠,٩٦	١٢١,٩٨
٤٥	11/G.5	٠,٩٥	١٦٧,٨١
٤٦	20/G10*	١,٠٨	٢١٤,٢٥
٤٧	20/G10 16spore	١,٠٦	٢٠٣,٩٤
٤٨	10cm/35 pigment	١,٠٨	٢٠٧,٤٣
٤٩	12/28	٢,٥٢	190.61
٥٠	12/34M	١,٩٧	88.30
٥١	11/G.S	١,٨٤	١٣٦,٤٥
٥٢	12/34m	٢,١٣	٨٥,١٩
٥٣	10cm/35pigment	١,٦١	١٣٢,٧٢

3.٢. Ability of *Streptomyces* isolates to produce hygromycin B

The ability of *Sreptomyces* isolates and the gene responsible for production of hygromycin B was performed by polymerase chain reaction technique (PCR) using sets of primers that amplify the gene. Only 3 of the 53 isolates tested showed positive results for the gene presence after analyzing on agarose gel as illustrated in figure (3-1) . Chaudhry and Rashid (2010) used two specific designed primers for

the hygromycin coding region to amplify a 670 bp fragment of this gene from genomic DNA. Hygromycin forward primer '5GCTCCATACAA GCC AACCAC-3' and reverse primer with 5'- CGAAAAGT TCGACAGCGTCTC-3' sequence was used for hygromycin amplification in this study.

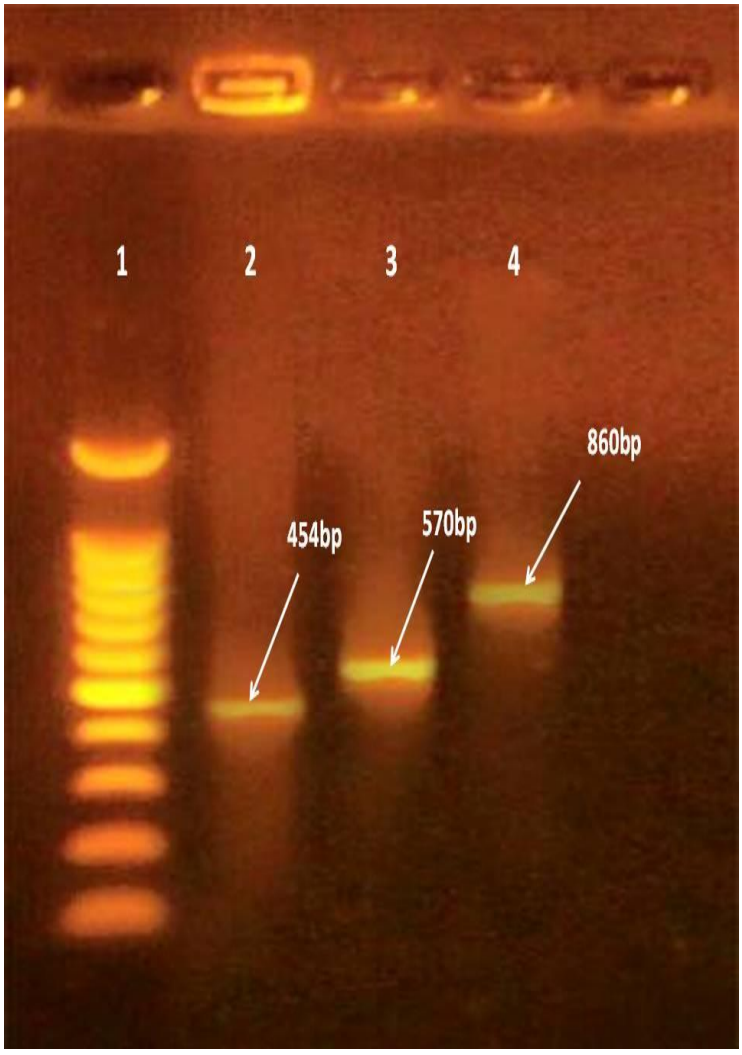


Figure (3-1) Agarose gel electrophoresis of PCR products of hygromycin B gene in *Streptomyces* isolates visualized under U.V. after staining with ethidium bromide at 80V for 1hour .

Lane 1:- 100bp DNA ladder marker

Lane 2:- product of Hygro1 primer

Lane 3:- product of Hygro2 primer

Lane 4:- product of Hygro3 primer

3.3. Antimicrobial activity of *Streptomyces* isolates:

Antimicrobial activity of the three isolates containing hygromycin B gene were tested by using agar well diffusion method and expressed as diameter of the inhibition zones against one Gram +ve bacteria (*Staphylococcus aureus*), five Gram–ve bacteria (*Pseudomonas aeruginosa , Proteus mirabilis, Escherichia coli , Klebsiella pneumoniae, Salmonella typhi*) and one yeasts (*Saccharomyces cerevisiae).* As shown in table (3-2), isolate symbolled "10cm/35pigment" of *Streptomyces* gave the highest antimicrobial activity against four of the seven test microorganisms (*S. aureus*, *Proteus mirabilis, S. typhi* and *Saccharomyces cerevisiae*) with inhibition zone diameters of 55, 70, 33 and 38 mm, respectively. While isolate symbolled "1/2/34" excreted its maximum antimicrobial activity against *E. coli* (60 mm) and Kleb**.** *pneumoniae* (12 mm**).** *Pseudomonas aeruginosa* was mostly effected by isolate "12/34m" of *Streptomyces* with an 18 mm inhibition zone. Adversely, lowest inhibition zone (9 mm) was recorded by isolate "12/34m" against the test bacteria *Kleb***.** *pneumoniae.*

From the above findings, it is obvious that isolate "10cm/35pigment" was the most efficient one among the three S*treptomyces* isolates when the first and the third highest inhibition zones (70 and 55 mm) were recorded by it. For such reason, this isolate was selected to be used in the following experiments of the research.

Table (3-2): Antimicrobial activity of three isolates of *Streptomyces* against seven test microorganisms including (G+ve bacteria, G-ve bacteria and a yeast).

Isolate	Inhibition zone diameter (mm)

symbol	*Staph. aureus*	*Ps. aeruginosa*	*Proteus mirabilis*	*E. coli*	*K.pneumoniae*	*Sal. typhi*	*Sacch. cerevisiae*
1/2/34	١٠	١٠	٣٠	٦٠	١٢	٣٠	٣١
12/34m	٢٢	١٨	٢٥	٣٣	٩	١٨	٢٣
10cm/35 pigment	٥٥	١٧	٧٠	٣٠	١٠	٣٣	٣٨

3.4 Identification of *Streptomyces* isolate10cm/35pigment:

3.4.1 Cultural characteristics:

Several diagnostic media were used to characterize the color of aerial mass and substrate mycelium, reverse-side pigment, melanin pigments, spore chain morphology and spore morphology. This isolate were examined by culturing on some standard identification media proposed by the International *Streptomyces* Project ISP (Shirling and Gottlieb, 1966) and by Pridham *et al*. (1957).

Table (3-3) which contains culture properties of the selected *Streptomyces* isolates on different media shows that the isolates grew well on yeast extract-malt extract agar ISP2 and inorganic salt-starch agar ISP4 after incubation for 10 days at 30˚C. The color of aerial mycelium was appeared light grayish, while the substrate mycelium appeared white. The reverse side of colonies was ranged from gray to colorless. These results came in accordance with those of the ISP description by Shirling and Gottlieb (1972) as referred to *Streptomyces hygroscopicus*. They described the mycelium *as* brownish grey or light greyish reddish brown (Grey color series) on ISP media 2, 3, 4, and 5. While the reverse side colors of the colonies were either colorless to greyish yellow, pale yellow, light olive brown or grey on ISP media 2, 3, 4 and 5.

Color series of the aerial mycelium established in the Bergey's manual of determinatives bacteriology (Buchanan and Gibbons, 1974) and in the category 4 of the Bergeys manual of systemic bacteriology, could be grouped in grey and white the series.

Cultural characteristics were also investigated on Glycerol–asparagines agar ISP5 pigment, Czapeck dox medium, Sabaroud maltose agar, Asparagine-dextrose–meat extract agar, Bennetts agar, PDA and nutrient agar after incubation for 14 days at ٣٠˚C (Janssen *et al.,* 2002). The cultural characteristics together with certain physiologic reactions were examined and recorded. The recorded growth was moderate to abundant on some of the media used as shown in figure (3-2 / A, B, C, D. E).

Cultural results of these isolates indicate that they belong to the *Streptomyces* genus (Tresner and Backus, 1956; Waksman, 1961; Shirling and Gottlieb, 1966; Holt, 1994).

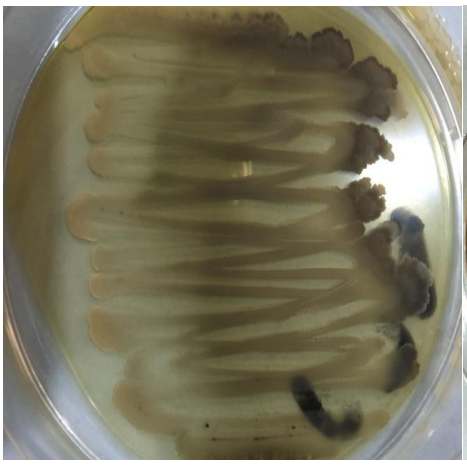
Table (3-3):- Cultural characteristics of *Streptomyces* isolates on different identification media.

medium	growth	Aerial mycilum	Sustrate mycilum	reverse side of colony	Diffusible pigment
ISP1	abundant	Whitish	Pale grayish	colorless	non
ISP2	abundant	Whitish to brown	brown	Light yellow	Yellow to light brown
ISP4	moderate	non	Light brown	Light yellow	non
ISP5	abundant	Whitish	Light gray	colorless	non

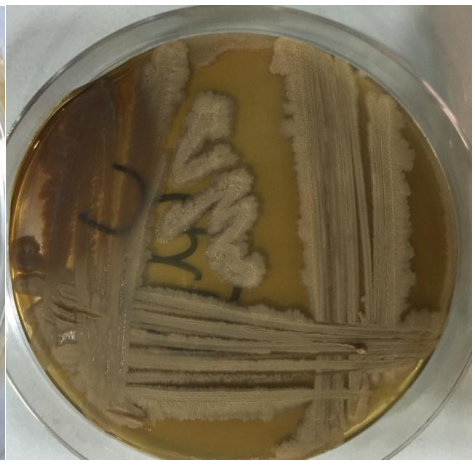
Czapeck dox medium	moderate	non	white	colorless	non
Sabaroud maltose agar	abundant	Whitish to brown	white	Colorless to gray	non
Asparagine-dextrose–meat extract agar	abundant	Whitish	White to gray	Colorless to gray	non
Bennetts Agar	abundant	gray	Brown to green	Light green	Light green

A

B



C



D

E

Figure (3-2) Growth of *Streptomyces* isolate10cm/35pigment on: A- Czapeck dox agar, B- Bennetts agar, C- PDA medium, D- ISP2 mediun and E- Nutrient agar.

3.4.2 Microscopic Characteristics:

Streptomyces isolate appeared after staining by Gram staining method as G (+), while the spore chain appeared as spirals. Morphology of the spore chains varied depending on the species, showing that they were straight and flexuous forms, like hooks with open loops and coils, which are usually used, among other features, to establish the differences between the isolate.

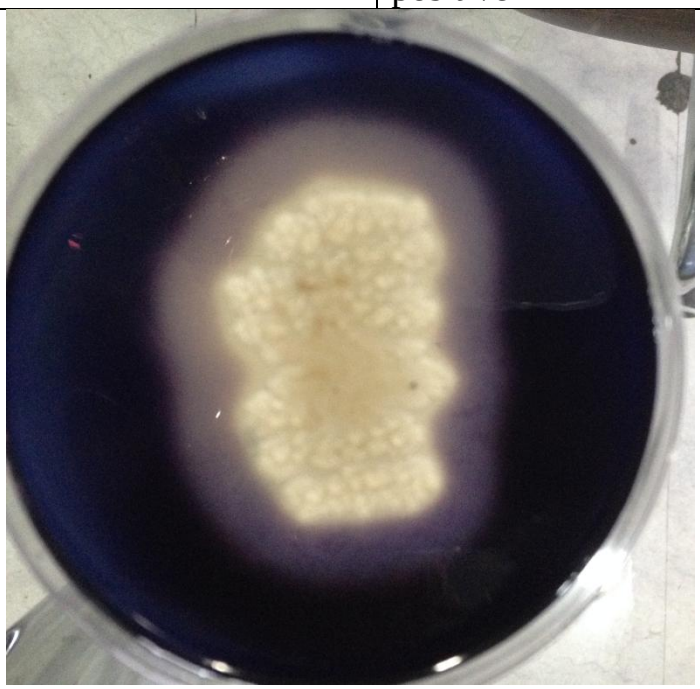
3.4.3 Biochemical Characteristics:-

As shown in table (3-4), *Streptomyces* isolate 10cm/35pigment gave positive results for: catalase, amylase which hydrolyze the starch (as shown in figure (3-3) and gelatinase, urease and utilization of citrate as the sole carbon source. Adversely, isolate 10cm/35pigment gave negative results for indole test and was also unable to lyse red blood cells and to produce melanin pigment. Results of carbohydrates fermentation indicate that the isolate was able to ferment (glucose, Inositol ,sucrose , fructose, Mannitol, and D-xylose) but unable to ferment L-arabinose **.**

Such findings are similar to those biochemical characteristics described by Parthasarathi *et al.*, (2012) and Biswas *et al.*, (2011) about *Streptomyces hygroscopicus*.

Table(3-4) Biochemical characterization of 10cm/35pigment *Streptomyces* isolate.

Characteristic	Result
catalase	positive
indole	negative
urease	positive
Starch hydrolysis	positive
Gelatin liquification	positive
Blood hemolysis	negative
Citrate utilization	positive
Melanin production	negative
Carbohydrates fermentation	
D-glucose	positive
Inositol	positive
L-arabinose	negative
Sucrose	positive
D-xylose	positive
Fructose	positive
Mannitol	positive



Figure(3-3) Hydrolysis of starch by 10cm/35pigment *Streptomyces* isolate10cm/35pigment grown on starch agar .

3.5 Amplification of hygromycin B phosphotransferase *hph* gene

Hygromycin B phosphtransferase was amplified by using two sets of specific primers , this primers were designated to amplify specific genetic element on the gene. PCR products of the gene were analyzed by gel electr -ophoresis technique to detect fragment amplification and compare it with 100bp DNA ladder marker. Figure (3-4) shows the amplified fragment at an accurate location supposed to be present where lane 2 represents the 323bp fragment while lane 3 represents the 327bp fragment. Dinga *et al*. (2011) used the hygromycin phosphotransferase (*hph*) gene as selectable marker in the binary vector pPEH after amplification using a set of primers hygSF and hygXR.

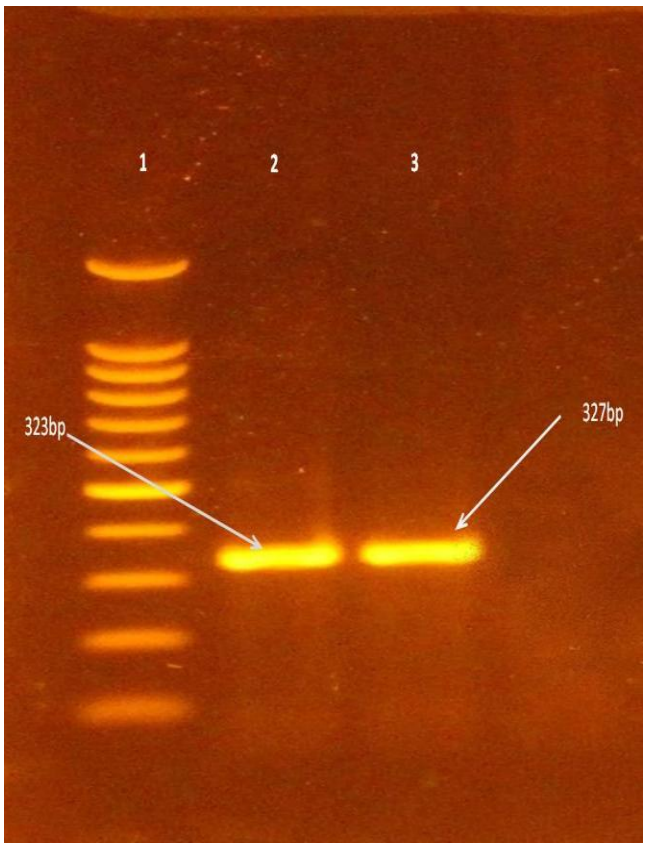


Figure (3-4) Agarose gel 1.5% electrophoresis of PCR products of hygromycin B gene(*hph*) gene in *Streptomyces* isolates at 80V for 1 hours.

Lane 1:- 100bp DNA ladder marker

Lane 2:- product of HYGN-1 primer

Lane 3:- product of HYGN-2 primer

3 ٦.Hygromycin B Separation by Ethyl acetate :

Hygromycin B is an atypical aminoglycoside which is a large and diverse class of antibiotic has a unique structural and functional properties produced by *Streptomyces* isolates10cm/35pigment (Borovinskaya *et al*, 1996)

Clear filtrate which obtained after propagation of *Streptomyces* isolate 10cm/35pigment in cultural broth was tested for presence and activity of antimicrobial compounds against the seven test microorganisms. Separation of the filtrate by ethyl acetate resulted into two phases; organic and aqueous**.**

Results showed that when hygromycin B standard (100mg/ml) was used, no any antimicrobial activity was appeared in the organic phase at concentration of 3mg/ml, while in the aqueous phase, good antimicrobial activity was recorded of crude extract as illustrated in table (3-5).

Hygromycin B is soluble in water, at concentrations >50 mg/ml, methanol, buffer solution or in ethanol, but it is practically insoluble in less polar solvents (Merck Index,1996). In the study of Plozza *et al*. (2011) , the aminoglycosides antibiotics were extracted from meat tissue or milk using an aqueous buffer.Also Sloltes (1999) stated that the aminoglycoside antibiotics (aminoglycosides) are hydrophilic molecules consisting of an aminated cyclitol associated with an amino sugar. Then, aminoglycosides are readily soluble in water, This result was disagree with that detected by Afifi ***et al*., 2012 that extract the hygromycin B in organic phase .**

Table (3-5):- Inhibition zone diameters (mm) form by hygromycin B ((crude aqueous extract(25mg/ml)) and hygromycin B ((standard solution (100mg/ml)) against the test microorganisms .

Test organism	Inhibition zone diameter (mm)

	Hygromycin B Standard(100mg/ml)	Aqueous Extract
Staph. aureus	٣٩	٢٧
Pseud.aeruginosa	38	٢٥
Proteus mirabilis	٤٥	٣٣
Kleb.pneumoniae	٣٧	٢٧
Salmonella typhi.	٣٣	٢٣
E.coli	٤٠	٢٦
Sacch.cerevisiae	٣٠	٢٢

3.7 Hygromycin B Detection by Thin layer hromatography :

Thin layer chromatography (TLC) technique was used in detection of the aminoglycoside hygromycin B. The mobile phase carried the spot of the sample through a stable stationary phase, and each compound had a specific affinity for the mobile and stationary phases, therefore, migrates at a different speed. One spot of the sample mixture migrates at same speed of the hygromycin B standard and same Rf value that is constant under stable conditions of chromatography. It could be possible to say that the proposed antimicrobial agent is to be belonged to hygromycin B (Touchstone and Joseph, 1983). Medina and Unruh (1995) also used thin chromatography method to separate and detect neomycin, gentamicin, spectinomycin, hygromycin B and streptomycin allowing multiresidue detection of these aminoglycosides. The respective RF values indicate the separation of these five compounds. This procedure provides a rapid and sensitive method for the semi-quantitative estimation of aminoglycosides. Hubicka *et al.*,(2009) stated that a TLC for identification and determination of amikacin, gentamicin, kanamycin, neomycin, netilmicin, and tobramycin (aminoglycosidic antibiotcs).

Figure (3-5) shows the TLC plate of crude extract of hygromycin B in the aqueous .solution. It could be seen in this figure the movement of the extract and the standard where both of them reach the same distance from the bottom .

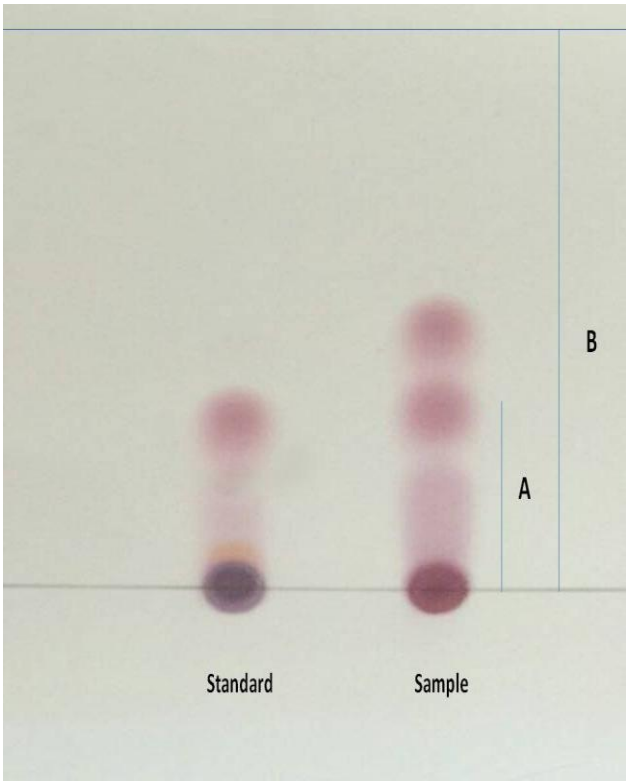


Figure (3-5): TLC analysis of crude extract hygromycin B produced by *Streptomyces* isolate 10cm/35pigment.

Rf = A/B

Rf for hygromycin B standard=2.5/7=0.357

Rf for sample =2.5/7=0.357

A= the distance from the starting point to the gravity center of the sample spot.
B = the distance from the starting point to the front of the developing solvent.

3. 8 Optimization of hygromycin B production conditions:

٣.8.1 Optimization of pH:

To investigate the effects of the initial pH medium on hygromycin B production, the *Streptomyces* isolate was grown in hygromycin B production broth medium with different pH values (4 , 5 , 6 , 7 , 8 , 9) before incubated at 30˚C for 7 days .

Results in table (3-6) show that the isolate has recorded their highest activities when the pH of the medium was adjusted to 8. At this pH, Highest antimicrobial

effect for hygromycin B was recorded with a (23) mm inhibition zone against the Gram negative bacteria *Proteus mirabilis.* Moreover, at this pH also, most efficient antimicrobial activity for hygromycin B against all three test organisms: *Staph aureus* (as Gram positive bacteria), *Proteus mirabilis* (as Gram negative bacteria) and *Saccharomyces cerevisiae* (as a yeast) was achieved when highest inhibition zone diameters (20, 23 and 18 mm, respectively) were recorded. Adversely, pH 4 led to the lowest antimicrobial activity for hygromycin B against all three test organisms with inhibition zones of (11, 11 and 9 mm, respectively).

Such results are closed to those obtained by Afifi *et al.*, (2012) when they found that the optimum pH for hygromycin B production was 8 and likely in agreement with result of Parthasarathi *et al.*,(2012) found that pH 7 showed maximum inhibition zone (26 mm) against *Kleb. pneumoniae* followed by *Ps. aeruginosa* (24 mm), *S. aureus* (20 mm), *B. subtilis* (19 mm) *and E. coli* (17 mm), in the substrate bombay rawa. Kavanag *et al.*,(1972) stated that , the assay of hygromycin B antimicrobial activity was more sensitive at pH 8.

The quantity of hygromycin B applied can be reduced by increasing the pH of the medium. At higher pH values, cells are more sensitive. The sensitivity of cells is pH dependent (i.e. the higher the pH of the culture medium the greater the sensitivity). Thus, the concentration of hygromycin B required for complete growth inhibition of given cells can be reduced by increasing the pH of the medium (Moazed, D. and Noller ,1987 Hemmi *et al.*, 1992)

Table (3-٦) Antimicrobial activity of hygromycin B produced by *Streptomyces* isolate 10cm/35pigment grown in production broth culture of different pH values for 7 days at 30˚C

pH / Test organism	Inhibition zone diameter (mm)					
	4	**5**	**6**	**7**	**8**	**9**
Staph. aureus	11	12	14	16	20	18
Proteus mirabilis	11	13	15	20	23	21

Sacch. cerevisiae	9	11	14	16	18	16

3.8.2 Optimization of growth temperature:

Streptomyces isolate was grown in the production medium after adjusting its pH to the optimum (8), then incubated the shaker incubator at different temperatures (25 , 30 , 35 , 40 , 45˚C) for 7 days.

Results in table (4-7) show that the *Streptomyces* isolate excreted its maximum biological activity when incubated at 35˚C, while less activity was detected at 45˚C. In this regard, Afifi *et al.* (2012) recorded 35˚C as the optimum temperature for hygromycin B produced from ***Streptomyces crystallinus* AZ151** Parthasarathi *et al.*,(2012) used (25°C, 28°C, 37°C and 50°C) as growth temperatures and found that 28°C was the optimum one to give maximum antimicrobial activity by producing zones of inhibition of; (24 mm) against *K. pneumoniae* followed by *P. aeruginosa* (17 mm), *E. coli* (16 mm), *S. aureus* (14 mm), *B. subtilis* (12 mm) in the substrate bombay rawa. In another study,

Oskay (2009) found the maximum zone of inhibition (18 mm) against S. *aureus* after incubation at 30°C temperature.

Table (3-7): Antimicrobial activity of hygromycin B produced by *Streptomyces* isolate 10cm/35pigment grown in pH 8 production broth culture at different temperatures for 7 days.

Temperature (°C) / Test organism	**Inhibition zone diameter (mm)**				
	25	**30**	**35**	**40**	**45**
Staph. aureus	17	20	23	18	17
Proteus mirabilis	20	23	28	25	19
Sacch. cerevisiae	13	15	18	16	12

3.8.3 Optimization of incubation period:

Optimum production of hygromycin B was also determined after incubation at different incubation periods (5, 7, 10, 12 days). Results in table (4-8) show that the maximum antimicrobial activty was obtained after 10 days of incubation at an optimum pH 8 and a temperature 35°C . Afifi *et al.* (2012) recorded the maximum production of hygromucin B also after 10 days of incubation while in the study of Parthasarathi *et al.*,(2012) the maximum zone of inhibition (24 mm) against *B. subtilis* after 10 days of incubation.

Table (3 -8)Antimicrobial activity of hygromycin B produced by *Streptomyces* isolate 10cm/35pigment grown in production broth culture at different incubation periods with an optimum pH 8 and an optimum temperature of 35°C.

Period(days) / Test organism	Inhibition zone diameter (mm)			
	5	**7**	**10**	**12**
Staph. aureus	17	20	23	18
Proteus mirabilis	20	23	28	25
Sacch. cerviciae	13	15	18	16

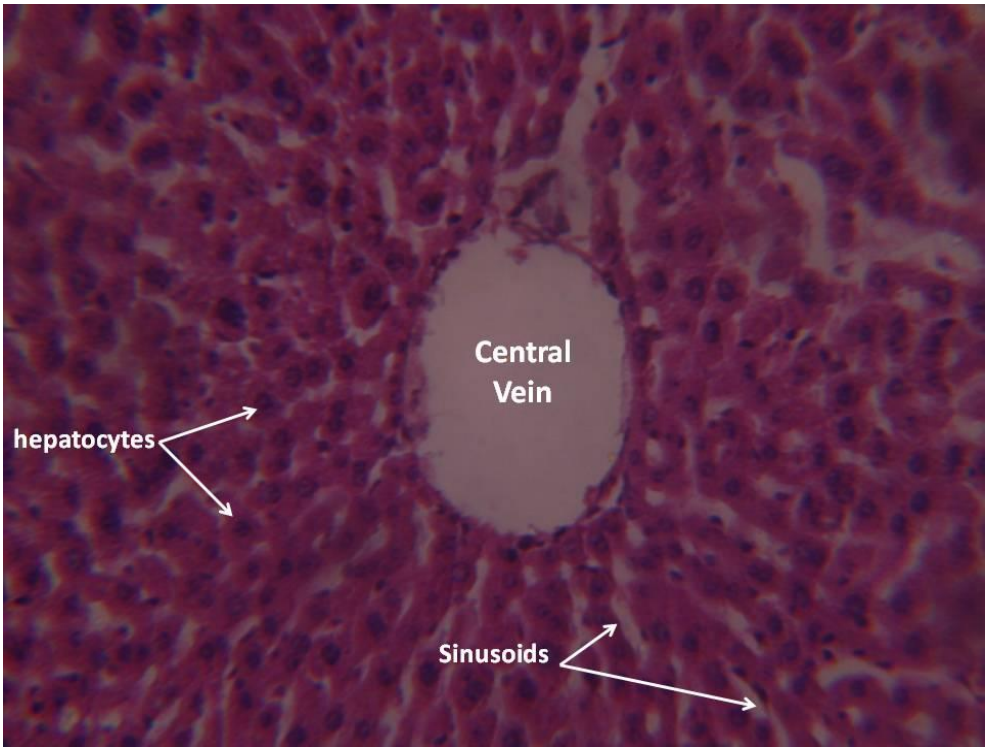
3.9Histological analysis of experimental mice:

Since liver is the foremost organ that receives all the materials absorbed by intestine through the portal vein, and it is the organ that must neutralize venoms, the toxic effect of most medications on liver manifest itself quicker than other organs. (Vahidieyerisofla *et al.*, 2014)

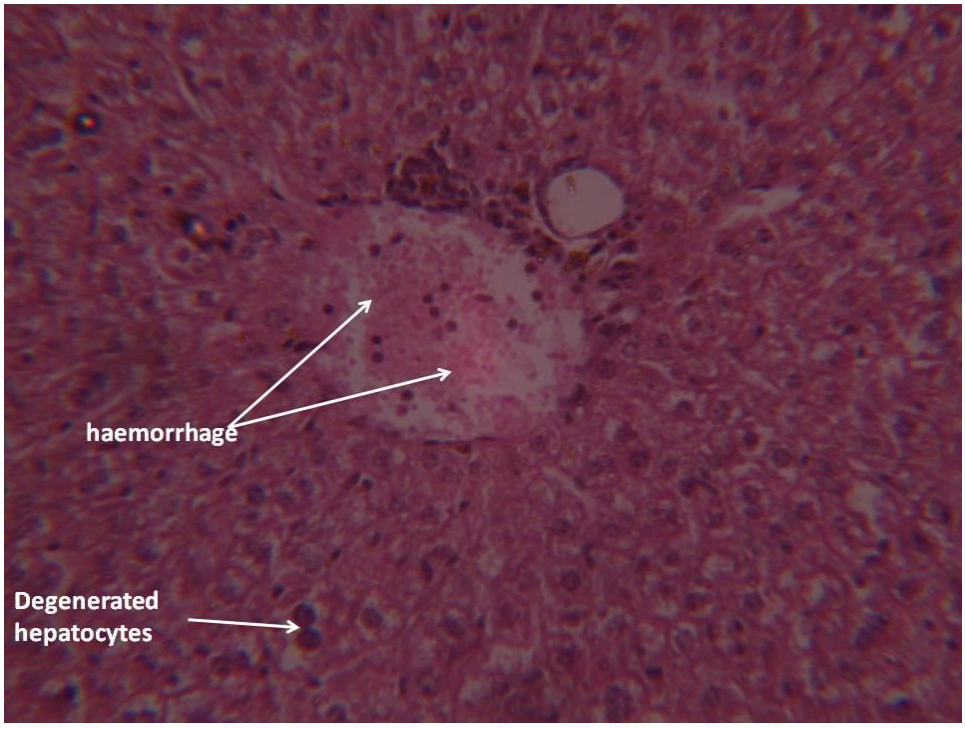
Oral addminstration with hygromycin B has been linked to mild effects which appeared after observing the microscopic slides of the liver tissue, of mice for both control and treated group . Microscopical examination of liver cross section slide of control mice showed normal structure, central vein,normal arrangement of hepatic cords, normal blood sinusoids and hepatocytes ,on the other hand the liver

section of the treated mice with hygromycin B extract observed little hemorrhage with infiltration of PMN cell and degeneration of hepatocytes as show in figure (3-6).

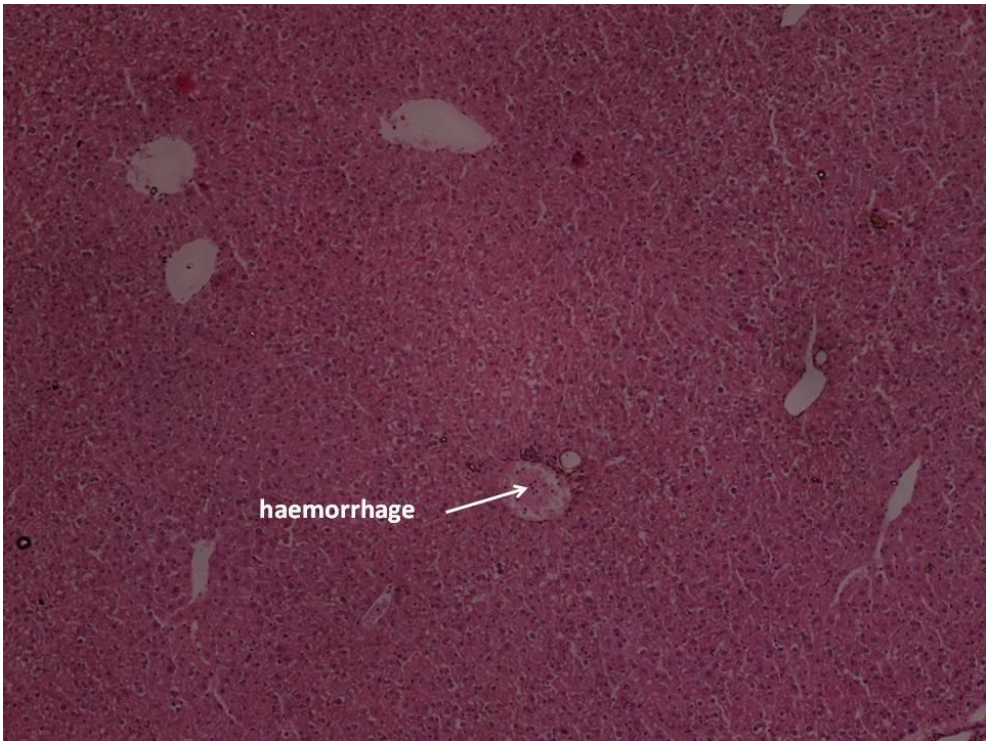
Khan *et al.,* (2011) stated that the administration with gentamicin belong the class of aminoglycosides had been also linked to mild and asymptomatic elevations in serum alkaline phosphatase levels, but rarely affects aminotransferase levels or bilirubin, and changes resolve rapidly once gentamicin is stopped. Only isolated case reports of acute liver injury with jaundice have been associated with aminoglycoside therapy including gentamicin, most of which are not very convincing. Recovery typically occurs within 1 to 2 months and chronic injury has not been described. Aminoglycosides are not listed or mentioned in large case series of drug induced liver disease and acute liver failure; thus, hepatic injury due to gentamicin and other aminoglycosides is rare if it occurs at all.



A



B



C

Figure (3- 6) A- Liver tissue sections of control mice showing normal structure, central vein (C.V.), normal arrangement of hepatic cords (H.C.), normal blood sinusoids (S) and hepatocytes., X 40x10. **B and C-** Liver tissue sectionof

Hygromycin B extract treated mice showing haemorrhage in the central vein, infiltration of PMN cell and degeneration of hepatocytes . at X 40x10 and X 10 respectively .

3.10 *Lactobacillus acidophilus* Identification:

3.10. 1 Microscopic examination:

Cells of *Lactobacillus acidophilus* appeared after staining by Gram staining as rods either single or in pairs but mostly grouped as chain. They were gram positive and non-sporeforming.

3.10.2 *Lb.* acidophilus API 50 identification:-

Lactobacillus strain was identified by using API 50 CHL,a standardized system, consisting of 50 biochemical tests . Grades of fermentation results 3, 4 and 5 were interpreted as positive (+) whereas 0,1 and 2 were negative (-). The isolate able to ferment Galactose, D-Glucose, D-Fructose, D-Mannose, Mannitol, N-Acetyl-glucosamine , Amygdalin, Arbutin, Esculin, Salicin, Cellobiose, Maltose, Lactose, Melibiose, Saccharose, Trehalose, D-Raffinose, Amidon, β-Gentiobiose, D-Turanose, D-Tagatose while negative to remain test (apendix1). Results indicate that the isolte was *Lactobacillus acidophilus* in return to idenetification table of the kit. Ozgun and Vural(2011); Mematu *et al.*,(2014) and Nigatu, A.(2000) also use the API 50 CHL kit as a taxonomic tool for *Lactobacillus* bacteria .

3.10.3 *Lb. acidophilus* molecular identification :

Lb. acidophilus was identified by amplifying To design specific and conserved primer(IDL22R 2079−2104) of 606bp predicted size(Kwon *et al.,* 2004) based on the 16S and 23S rRNA genes then PCR products of the gene were analyzed by gel electrophoresis technique to detect fragment amplification as shown in figure (3-7)

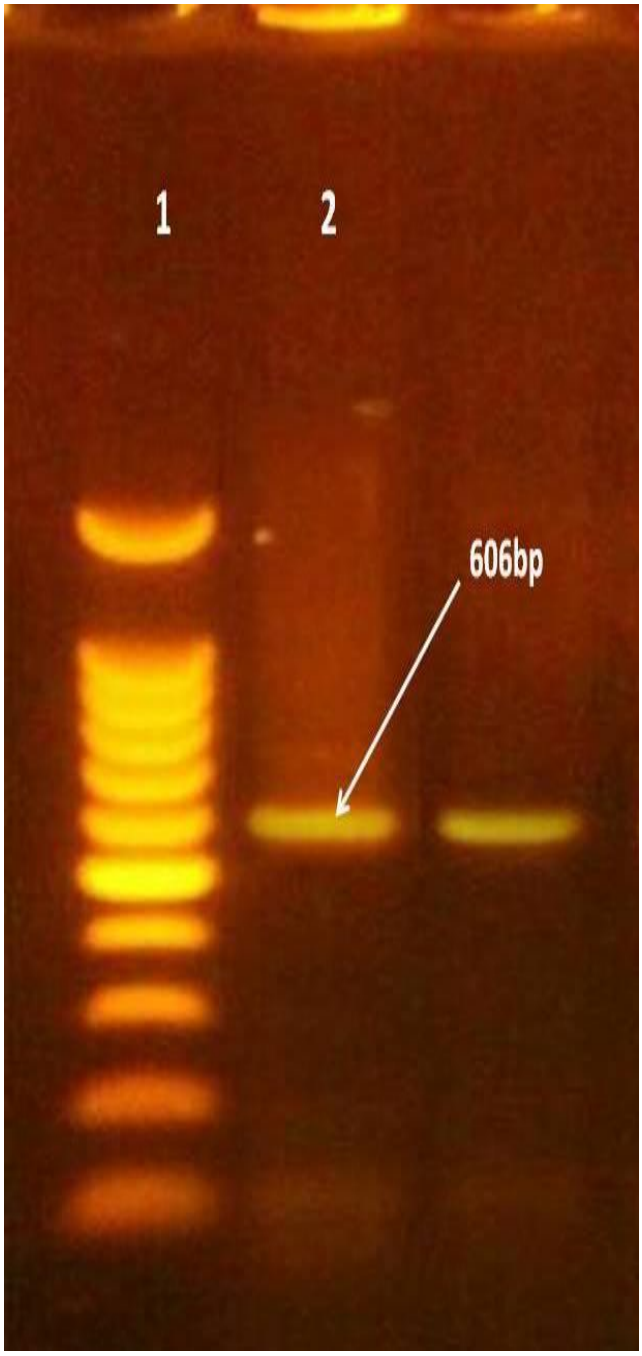


Figure (3-7): Agarose gel electrophoresis of PCR products of 16s rRNA gene in *Lactobacillus acidophilus* bacteria.

Lane 1:- 100bp DNA ladder marker

Lane 2:- product of IDL22R 2079−2104 primer

3.11 Plasmid extraction:

Plasmid DNA (pTKIP) 4491bp was extracted from *E.coli* bacterial culture as method described by addgene/USA then analyzed in 0.7% agarose gel for 2 hours and 60 voltage . Since the movement of the DNA fragment depended on their molecular weight and agarose pores , low concentration of agarose was used to let the high plasmid size to migrate along the gel. Figure (3-8) show the movement of the plasmid across the gel

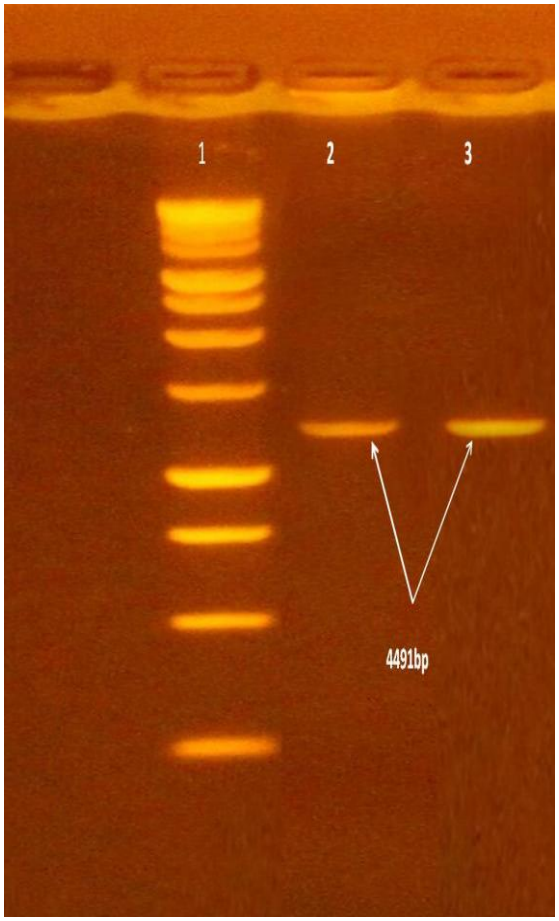


Figure (3-8): Agarose gel (0.7%) electrophoresis of extracted (pTKIP) plasmid DNA from E.coli , for 2 hours and 60 voltage .

Lane 1:- DNA ladder marker

Lane 2:- plasmid DNA of 4491bp

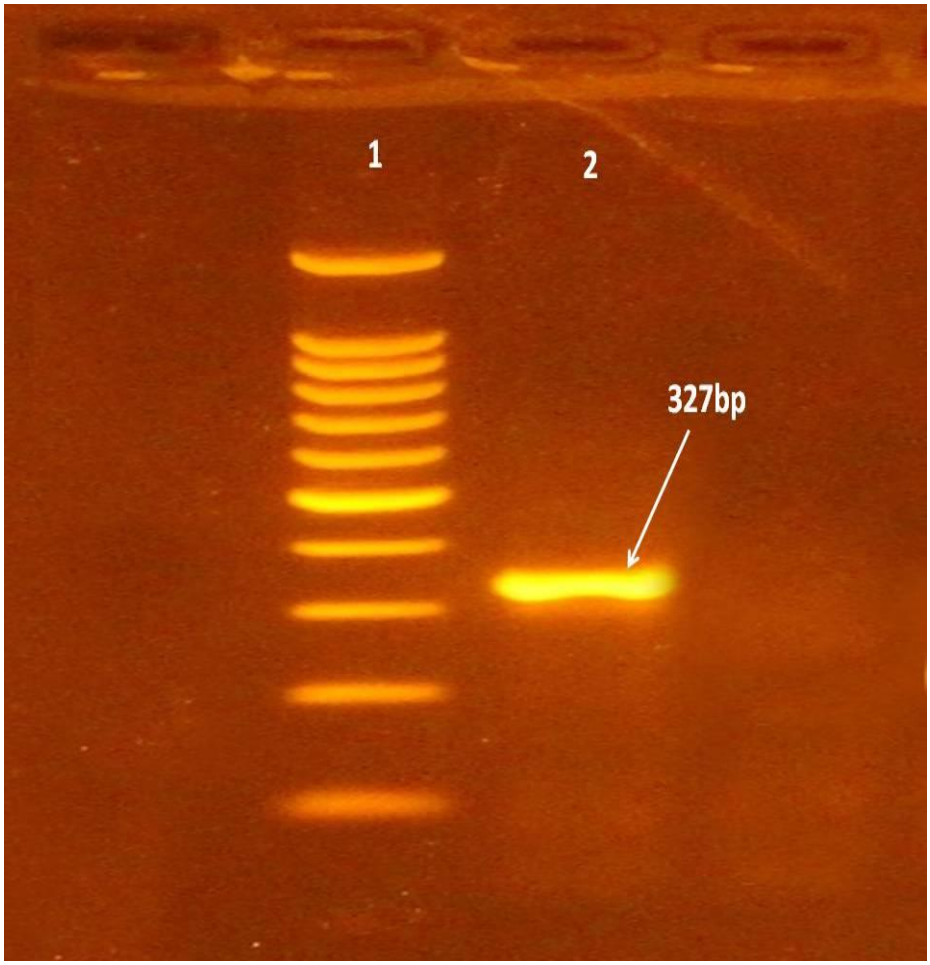
3.12 Transformation of *Lb. acidophilus* by electroporation

Transformation of *L. acidophilus* yielded 17.5 x 10^8 cfu/ 100 ng plasmid DNA used in ligation mix when grown on MRS medium with 100µg/ml hygromycin B . Proper assemble of recombinant plasmids was confirmed by colony PCR using

specific designed primers(HYGN-2 previously used) for hygromycin B phsphotransferas (*hph*) gene assembled in the plasmid , then PCR products of the gene were analyzed by gel electrophoresis technique to detect fragment amplification and compare it with 100bp DNA ladder marker as shown in figure (3-9).

Electroporation is being used as one of the easiest methods to perform a genetic transformation. Harlander and McKay (1984) reported a successful application of electroporation to streptococci, and then a widespread interest in the possibility of bacterial electro transformation had been prompted. Scheirlinck *et al.* (1989)and Gory *et al*. (2001) also used electoporation successfully for transformation of *Lactobacillus plantarum* , and other Lactic acid bacteria (LAB).

In The study of Kim *et al.*,(2005) the electroporation technique also used to trasform *Lactobacillus acidophilus* ATCC 43121 and obtained a significant transformation efficiency. Results of Spath *et al.,* (2012) showed Best transformation efficiencies were obtained using the strain *L. plantarum* CD033 and non-methylated plasmid DNA. Thereby achieved transformation efficiencies of ~ 109 colony forming units/µg DNA in L. plantarum CD033. Lactic acid bacteria (LAB) play an important role in agricultural as well as industrial biotechnologyo and therefore need to development of improved LAB strains.



Figure(3-9):- Agarose gel 1.5% electrophoresis of PCR products of hygromycin B resistance gene (*hph)* of the recombinant plasmids in *Lactobacillus acidophilus* transformed cell , at 80V for 1hour.

Lane 1:- 100bp DNA ladder marker

Lane 2 and 3 :- product of HYGN-2 primer

CHAPTER FOUR

CONCLUSIONS AND RECOMMENDATIONS

4.1 Conclusions :-

1- The hygromycin B gene can be detected rapidly using specific primers designed for this purpose.

2- From a total of 53 *Streptomyces* isolate only three have the ability to produce Hygromycin B antibiotic .

3- The *Streptomyces* isolate producing hygromycin B antibiotic is belong to *hygroscopicus* species.

4- For this 10cm/pigment *Streptomyces isolate , hygromycin B production media with pH 8* and incubation at 35˚C for 10 days could be used as an optimum condition for hygromycin B production.

5- Bacterial strain that produce hygromycin B has a wide range of biological activity.

6- Hygromycin B was found to cause a mild effects on mice liver.

7- Electropraton is an effective tool for transformation that give $1.75x10^{8}$ cfu/100ng plasmid DNA transformation efficiency .

4.2 Recommendations :-

1- Using the transformed *Lactobacillus acidophilus* as an additive for life stook feed.

2- Using hygromycin B for pre immunization fo farm animals against lethal disease.

3- Studying the staibility of the cloning vector used in this study and the level of expression .

4- optimize the parameters effect on the electroporation efficiency .

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No.	Carbohydrates	Results	No.	Carbohydrates	Results
1	Glycerol	0	**26**	Salicin	90
2	Erythritol	0	**27**	Cellobiose	90
3	D-Arabinose	0	**28**	Maltose	80
4	L-Arabinose	5	**29**	Lactose	80
5	Ribose	2	**30**	Melibiose	15
6	D-Xylose	0	**31**	Saccharose	100
7	L-Xylose	0	**32**	Trehalose	85
8	Adonitol	0	**33**	Inulin	0
9	®-Metil-D-xiloside	0	**34**	Melezitose	0
10	Galactose	80	**35**	D-Raffinose	30
11	D-Glucose	100	**36**	Amidon	30
12	D-Fructose	100	**37**	Glycogen	0
13	D-Mannose	97	**38**	Xylitol	0
14	L-Sorbose	0	**39**	β-Gentiobiose	100
15	Rhamnose	0	**40**	D-Turanose	7
16	Dulcitol	0	**41**	D-Lyxose	0
17	Inositol	0	**42**	D-Tagatose	50
18	Mannitol	50	**43**	D-Fucose	0
19	Sorbitol	0	**44**	L-Fucose	0
20	α–Methyl-D-mannoside	0	**45**	D-Arabitol	2
21	α–Methyl-D-glucoside	0	**46**	L-Arabitol	0
22	N-Acetyl-glucosamine	100	**47**	Gluconate	0
23	Amygdalin	75	**48**	2-Keto-gluconate	0
24	Arbutin	75	**49**	5-Keto-gluconate	0
25	Esculin	100	**50**	Gas production from glucose	0

الملخص

للتحقق من قدرة عزلات الـ *Streptomyces* لأنتاج مضاد الـ Hygromycin B كمضاد للديدان في تغذية الدواجن ، واختيار ناقل تعبيرمناسب يشفر لجين المقاومه ، من مجموع ٥٣ عزله من بكتريا الـ phosphotransferase Hygromycin B و أختباره للتحري عن وجود مورث الـ DNA ، أستخلص الـ *Streptomyces* . اعطت ثلاث عزلات فقط PCR بأستخدام بادئات متخصصة في تقنية الـ Hygomycin B منها نتيجة موجبة لوجود المورث المسؤول عن انتاج هذا المضاد الحيوي. و لأختيار العزلة الاكثر كفاءه، فقد أخضعت العزلات الثلاث هذه لفحص تقييم الفعالية الحيوية. بعدها شُخصت العزلة الأكفأ مظهرياً وكذلك بأستخدام الفحوصات الكيموحيوية.

كُشِفَ عن المورث المسؤول عن الـ hygromycin B phosphotransferase (*hph*) hygromycin. وعند استخلاص ال PCRبأستخدام بادئات متخصصة مصممة في تقنية ال بأستخدام خلات الاثيل، فُصل المحتوى العضوي عن المائي في راشح المزرعه البكتيرية B السائلة، واعطى المحتوى المائي فقط فعالية حيوية بأستخدام تقنية الأنتشار في الحفر على سطح عند تركيز٢٥ملغم/مل (كمستخلص خام) ، (Agar well diffusion technique)الأغر اعطى هذا الطور فعالية حيوية ضد مجموعة من الاحياء المجهرية اشتملت على بكتريا واحدة وخمس سالبة لغرام (*Staphylococcus aureus*)موجبة لغرام *Pseudomonas aeruginosa)*، *Proteus mirabilis,*، *Escherichia coli*، *Klebsiella pneumoniae* ، *Salmonella typhi)و خميرة واحدة((Saccharomyces cerevisiae*.

عند الكشف عن ال hygromycin B aminoglycoside بأستخدام كروموتوغرافيا (R_f) ٠,٣٥٧الطبقه الرقيقه لتأكيد وجود المضاد الحيوي ، تم الحصول على نفس معدل الجريان القياسي .hygromycin Bللـ

اظهرت نتائج الظروف المثلى للوسط الزرعي البكتيري ان اعلى فعالية حيوية للـ hygromycin Bم ٣٥ والحضن بحرارة ٨ والرقم الهيدروجيني ٥ تم الحصول عليها عند لمدة ١٠ ايام.

على كبد الفئران المختبرية وأظهرت hygromycin B تمت دراسة التأثير السمي للـ تغيرات طفيفه على انسجة كبد الفأر.

من عينة لبن وشخصت بالطرق *Lactobacillus acidophilus* عزلت بكتريا الـ صُمِمَ ناقل كلونة 16S ribosomal gene. وتقنية.API 50 kitالتقليدية وكذلك بأستخدام الـ لأستخدامه في تحول خلايا بكتريا الـ *E.coli* وعزل من بكتريا (*hph*)يحتوي على مورث الـ وأعطت كفاءة تحول Electroporation بواسطة تقنية الـ *Lactobacillus acidophilus* من الدنا البلازميدي. أختبرت الخلايا المحولة بأستخدام ng $١٠^{٨}$ مستعمره/١٠٠xبمقدار ١,٧٥ واظهرت وجود المورث في نتائج الترحيل الكهربائي (*hph*)بادئات متخصصة للكشف عن مورث الـ

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وزارة التعليم العالي و البحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الاحيائية

تحول بكتريا الـ *Lactobacillus acidophilus* بجين الـ Hygromycin الناشئ من بكتريا الـ *Streptomyces hygroscopicus*

أطروحة

مقدمة الى مجلس كلية العلوم / جامعة النهرين
كجزء من متطلبات نيل درجة دكتوراه علوم فلسفة في التقانة الأحيائيه

من قبل
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شباط ٢٠١٦

جُمادى الاولى ١٤٣٧