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Research Article

MOLECULAR ANALYSIS OF BACTERIAL GENE CODING CHITINASE ENZYMES, FAMILY 19 STREPTOMYCES

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ABSTRACT

Fungal phytopathogens pose serious problems worldwide in the cultivation of economically important plants.

Chemical fungicides are extensively used in current agriculture.However, excessive use of chemical fungicides in agriculture has led to deteriorating human health , environmental pollution, damaged to ecosystem and development of pathogen resistance to fungicide.

Because of the worsening problems in fungal disease control , a serious search is needed to identify alternative methods for plant protection, which are less dependent on chemicals and are more environmentally friendly. Microbial antagonists are widely used for the biocontrol of fungal plant diseases. Many species of actinomycates, particulary those belonging to the genus sterptomyces, are well known as antifungal biocontrol agents that inhibit several plant pathogenic fungi.

Another way biological control has been developed as an alternative of chemicals to tock with plant pathogenic fungi. Considering high presence of chitin in fungal cell wall, chitinase enzyme is camped as an effective biocontrol agent against phytopathogenic fungi. Streptomyces bacteria are able to produce various

chitinase enzymes, chitinases produced by streptomyces belong to the families 18 and 19 glycosyl hydrolases. The antifungal activity is mostly shown by fomily 19 Chitinases. In comparison with bacterial family 18 chitinases, the specific hydrolyzing activity of chitinase 19 against soluble and in soluble chitinous substrates has been markedly higher. Considering the importance of family to investigate antifungal potential of streptomyces bacteria isolated from east Azarbijan region soils based on molecular identification of family 19 chitinase. encoding gene in these bacteria.

To aim the purpose 110 soil samples were collected from East Azarbaijan and 310 strepomyces isolates were selected using macroscopic and microscopic observations. DNA genomic of all of the isolates were extracted and PCR reactions was done using chitinase 19 designed primers as marker.

Totally isolates were selected with molecular selection and antagonistic test were done. One of the isolates exhibit the most strong antifungal activity.

The strain was identified using 16srDNA gene, and the chitinase encoding gene were amplified partially to prove the PCR selection. Finally the bacterium were introduced as potentially biological fertilizer.

Keywords:streptomyces, family 19 chitinase, antifungal activity,16srDNA gene, biological control.

INTRODUCTION

Today, with the widespread use of chemicals in gardens and fields leading to the eradication of pathogens, there is a risk of insects and beneficial microorganism's destruction. Reducing environmental pollution caused by chemical pesticides led to new insights into the environmental control system and its upgrade. Practical use of biological control agents by reducing the survival of the business processes or the use of biological agents in the field is limited. So, it is important to screen for beneficial microorganisms. Streptomyces species are G^+ bacteria, standstill and filamentous bacteria which are located among environmental control factors. Streptomyces colonies are recognizable with chalky appearance and their smell like the soil. They are widespread in the nature and specially live in the soil and are very important parser. Streptomyces species are metabolically capable of metabolizing many different compounds including sugars, alcohols, amino acids and cyclic compounds by hydrolytic enzymes. Also a species of Streptomyces in soil salinity tolerant plants to stress and is involved in the control of fungal diseases (3,4). Fertilization and plant disease control is the power of Streptomyces bacteria that enter the soil to increase tolerance of plants against pests and diseases, and it can be replaced chemical fertilizers, and pesticides. The bacteria can also inhibit soil-borne fungal disease causing decisive effect on crop production has increased (1, 4).

One of the ways to control diseases caused by pathogenic fungi that have no adverse effects is the use of biological control methods. Unlike synthetic materials, the microbiological substances that are less toxic species have been effective, readily biodegradable and have low allergenic. In addition, this material does not

accumulate in food products and are also inexpensive and suitable for use on an industrial scale. Actinomycetes, particularly Streptomyces species are G^+ bacteria, many of which are soil-borne, broad-spectrum biological control agents such as antibiotics, hydrolytic enzymes such as chitinase and enzyme inhibitors against fungal pathogens, plants produce and secrete (7). Most of the other Streptomyces species in the temperature range 15 to 37 will grow. Furthermore, Streptomyces performance including different mechanisms, such as inhibition of pathogens by producing antibiotics, competition for iron through siderophore production, chitinase, glucanase and other sodium compounds such as phenyl acetic and phenyl acetic is suitable. The useful features of Streptomyces have attracted many researches for separation and recovery of this bacterium in biological control programs (2,3).

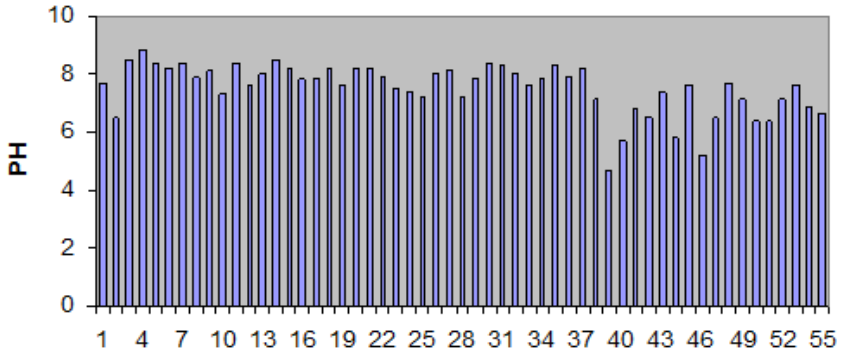
Streptomyces have direct antifungal activity, which has been previously reported. Reverse the osteolytic activity of these bacteria mainly is as a result of hydrolyses enzymes chitinase and glucanase (3). Chitin is a major component of fungal cell walls and is one of the most important features of the Streptomyces chitinase enzyme substrate capable of using chitin as a carbon source. Due to the high percentage of chitin in the cell walls of fungi, chitinase enzyme is considered as a biological control agent effective against fungi PhytoPathogen. The Streptomyces bacteria are able to produce various kinds of chitinase enzymes. Chitinase enzyme produced by Streptomyces belonging to two families 18 and 19 of qlycosil hydrolyses (5,6). Family 19 indicate anti fungi activities. Azerbaijan Shargi due to the cultivation of cucurbits and vegetable production is a major province in the country. So we should consider seriously the soli diseases *phytophthora* sp.44D, *Fusariumsolani* in order to increasing function and have careful information to control the disease.

MATERIALS AND METHODS

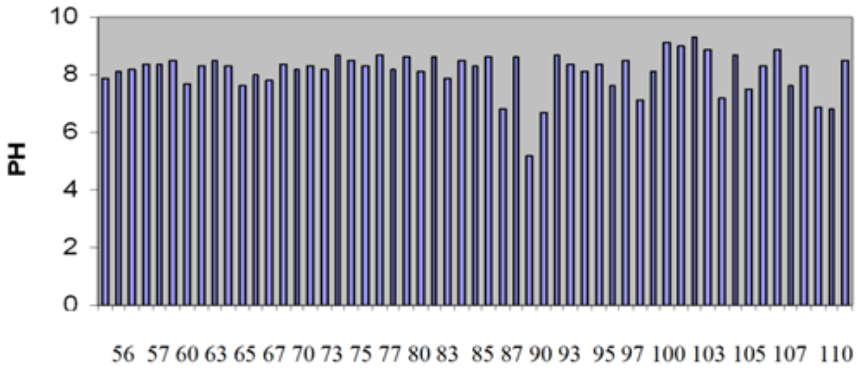
A total of 110 soil samples from a depth of 30-15 cm in spring and summer soil were collected from different areas of East Azarbaijan province. The soil samples were isolated from the acetone mists. Isolated from a soil sample dilutions were prepared according to $10^{-3}, 10^{-2}, 10^{-1}$. Investigation showed that the second was the best dilution to isolate the cause of the suitability of potential microbial concentration in the dilution. After planting soil diluted the sample isolate 310 was isolated microscopic and macroscopic observations. PH of soils in different areas of the conclusion was that 94 (85%) of the samples have a pH above 7.

PH	Name of the area of sampling	Number of sampling	Number of picked up samples	Row	PH	Name of the area of sampling	Number of sampling	Number of picked up samples	Row
7.9-8.4	Shahverdi	9	56-64	8	7.9-8.5	Tabriz	8	1-8	1
7.3-8.4	Mountain of Sahand	13	65-77	9	7.8-8.4	Jolfa	6	9-14	2
8.1-8.9	Shabestar	6	78-83	10	8.2-8.5	Kaleibar	7	15-21	3
7.1-8.5	Mountain of Misho	10	84-93	11	7.9-8.6	Ahar	8	22-29	4
7.8-8.3	Marand	4	94-97	12	9-9.3	Malekan	15	30-44	5
7.2-8.9	Bonab	5	98-102	13	8.9-9	Azarshahr	6	45-50	6
6.5-8.7	Heris	8	103-110	14	8.1-8.5	Khodaafarin	5	51-55	7

Table 1: The sampled areas of the soil



Soil Samples (a)



Soil Samples(b)

Figure 1: East Azarbaijan region soils pH, a) number 1 to 55, b) number 56 to 110

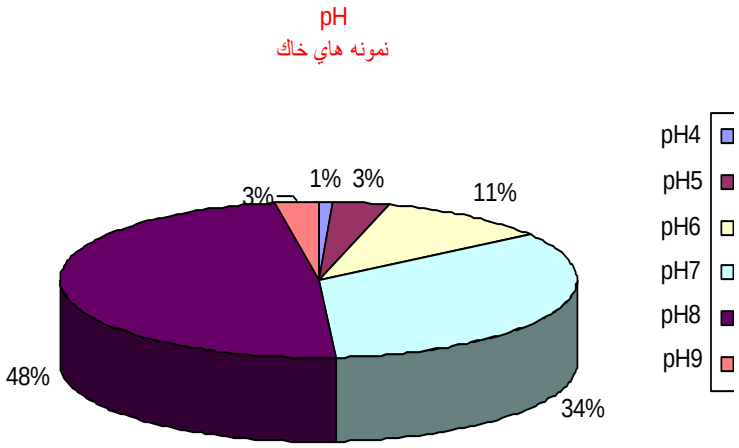


Figure 2: pH percent of evaluated soil samples.

Seven days after the second dilution culture plate colonies were counted.

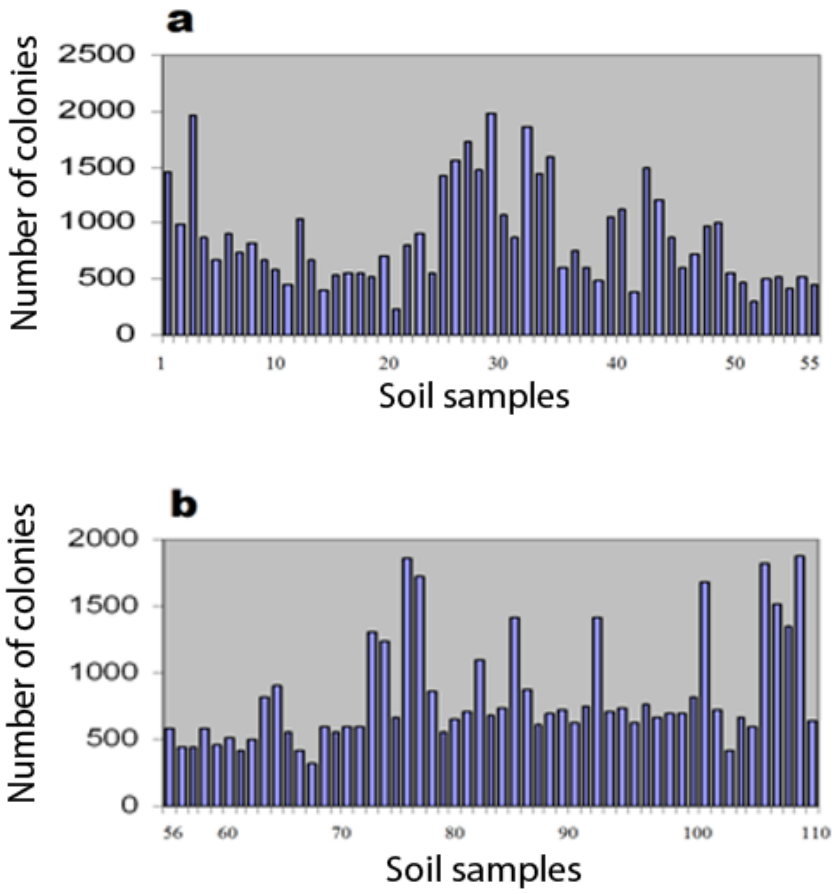
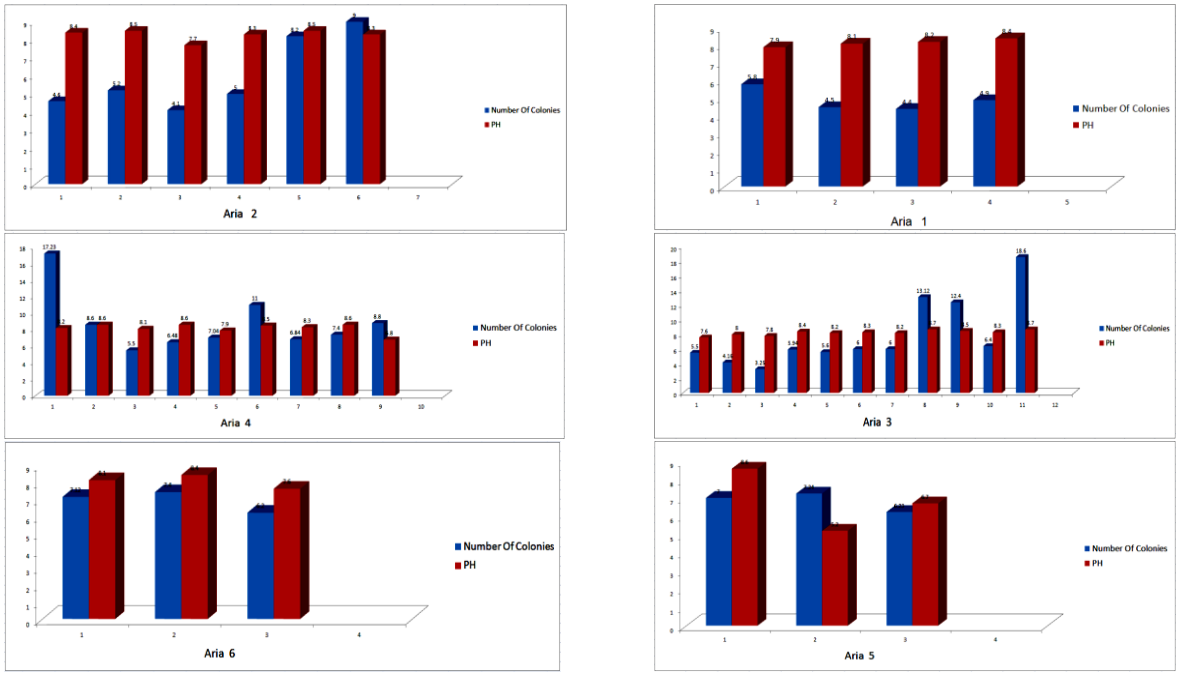


Figure 3: Colony numbers from soil samples, a) number 1 to 55, b) number 56 to 110.



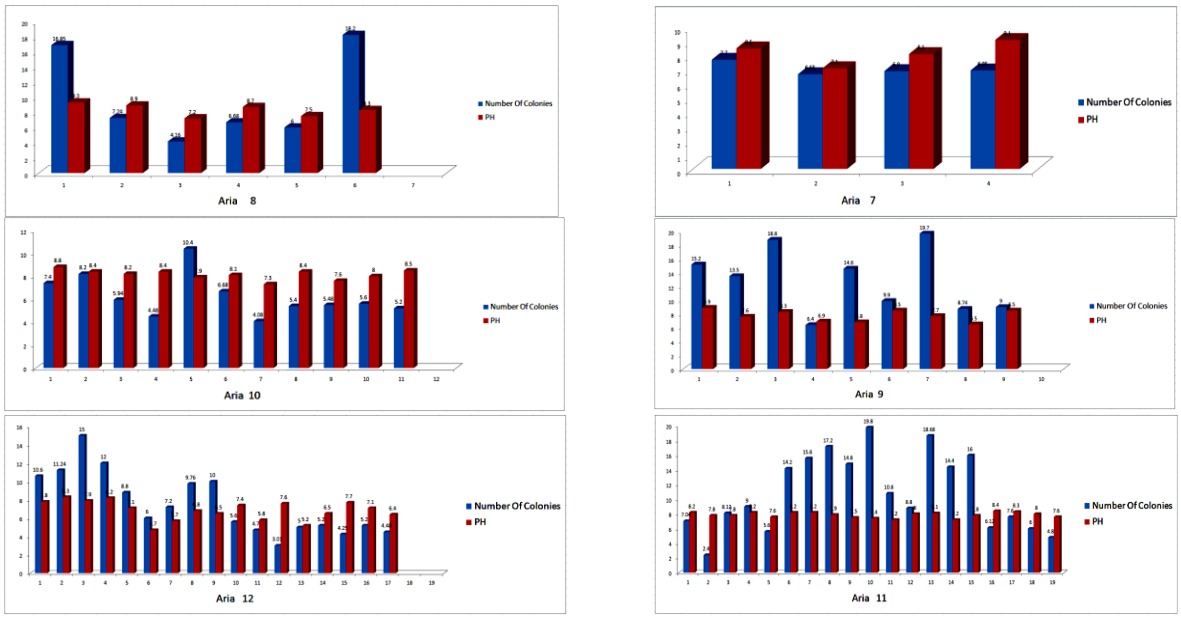


Figure 4: Graphs to compare the effect of pH on colony.

According to the above mentioned graphs areas with high alkaline pH have more Streptomyces species. In other words, alkaline soils have high potential to isolate the bacteria. For long-term storage of bacteria isolated in this study were used frozen bacteria in -80°C. Genomic DNA extracted from the bacteria Streptomyces wall peptide glycan very hard and resistant to harsh methods impossible; Therefore, using the fierce heat shock applied to alternate freezing and thawing, the extract was then broken wall Peptido glycan bacteria. Both direct and reverse sequences were done via BLAST 2 Sequences software. After obtaining the complete sequence of the fragment of the nucleotide sequence of the gene sequence databases by bioinformatics software were compared with Global Biotechnology (NCBI: National Center for Biotechnology information). BLAST results showed that 100% of these sequences show overlap with the highest similarity (96%) of 19 bacteria Streptomyces griseous chitinase enzyme coding gene sequences.

RESULTS AND DISCUSSION

Genomic DNA extraction:

Due to the fact that the Peptido glycan walls of Streptomyces bacteria are very hard and resistant, the conventional methods for cell lubrication are not enough for its lubricating and destroying. So, via severe heat shock, alternate freezing and thawing, at first the Peptido glycan wall was broken and then the rest of the steps have been done. Figure 1 shows the extracted genomic from the isolated bacteria with above mentioned method. DNA concentration of extracted genomic was estimated by size marker comparison on the Agarose gel. The best pure number was closer to 30 nano grams per micro liter.

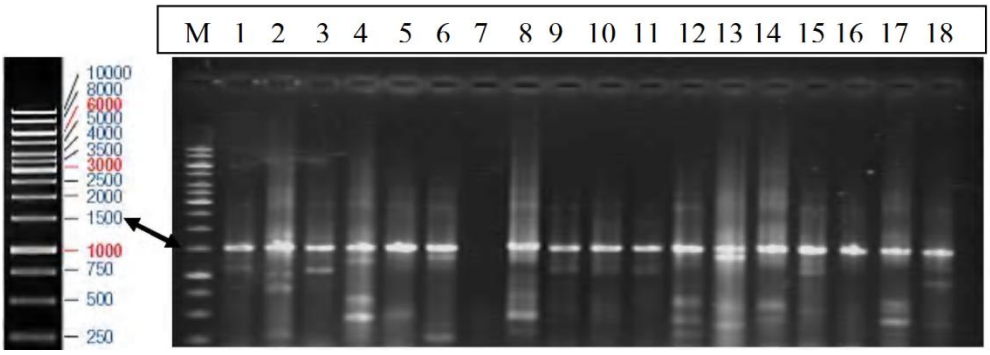


Figure 1: Genomics extracted from the Streptomyces bacteria: size marker, 1kb, DNA ladder.

Family 19 Chitinase enzyme coding gene amplification using PCR method:

Primers used in 2005 by Hooster et al, which have been used in the PCR, were designed based on amplification of a fragment of 885bp, coding gene, from Streptomyces griseus bacteria, Chitinase enzyme.

In this research, the chain polymerase reaction has been done via the bacteria genomes and CH19F/CH19R starting pair were used as DNA pattern in order to study the amplification of the fragment of 19 family Chitinase enzyme' coding gene. The results revealed that among 310 tested isolates, just 5 cases contained this gene. Their amplification fragments size was between 750 to 1000 bp (figure 2).

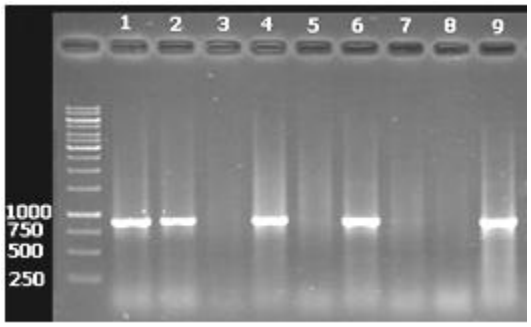


Figure 2:PCR results of 885bp fragment amplification of 19 family Chitinase enzymes with CH19F/CH19R starting pair. Size marker: 1Kb DNA Ladder (left).

Cloning of the family 19 chitinase gene amplified fragment:

The cloning, not only increases the quality of reading sequence, but also makes it possible to preserve the corresponding DNA segment within the vector for a long time. Also, via providing transgenic bacteria, we can multiply the Vector and DAN segment and use it in subsequent studies.

Fusion reactions:

Two no adherent DNA in the absence of hydrogen bonds is less efficient and only is possible with T_4 DNA Ligase. Due to the low temperature of the hydrogen bonds, executed by a nucleotide binding is not stable enough. Therefore, the reaction is preferably performed at 4◦ C and long time (12 to 16h).

Host cells transgenesis making:

Due to the special specifications of E.coli bacteria walls, the heat shock method causes some pores in E.coli plasmas for a short time and makes DNA to enter the cell. After removing the shock, the pore closes again. It seems that as a result of E.coli cells treatment with $CaCl_2$, cell membrane negative charges are neutralized by calcium cations, temporarily and increases the positive charges of the cell surface. So, the DNA molecule with negative charge gets inside the cell, without being repelled by negative charges of membrane. Under no circumstances or other chemicals, the cells don't get compatible (8).

Screening of transgenic colonies:

The transgenic cells were transferred to selective medium containing Ampicillin, IPTG, and X-Gal and incubated (as in Figure 3), both blue and white colonies were established on the environment. The blue colonies showed the cells with no recombinant plasmid. So, the white colonies were selected as the correct transgenesis cells and cultured in LB liquid environment containing Ampicillin and stoking for long time preservation.

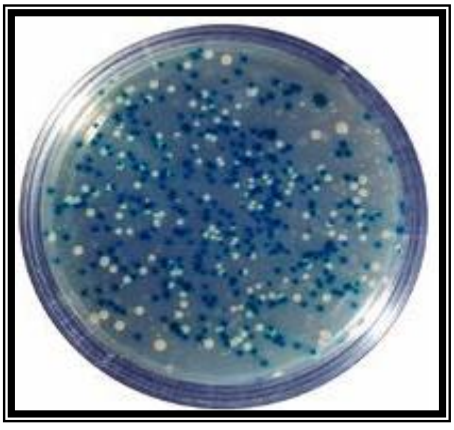


Figure 3:the white and blue colonies on the selective culture medium for screening of transgenic colonies.

Recombinant plasmid extraction:

The recombinant plasmid extraction from the cultured white colonies in LB liquid containing Ampicillin has been done via alkaline lubrication. Its product was cleared via Electrophoresis on the

Agarosegel(Figure 4).

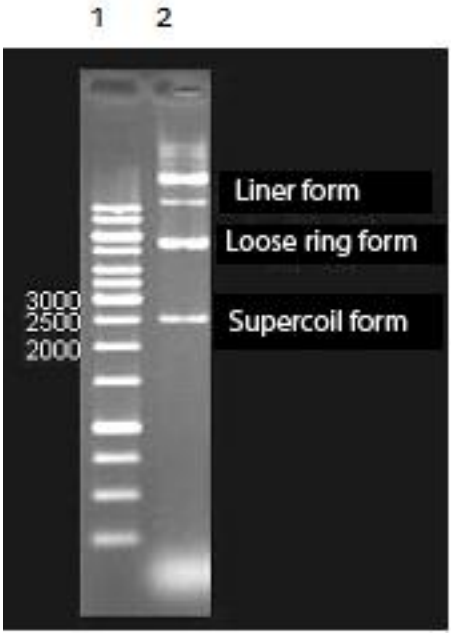


Figure 4:the recombinant plasmid extraction containing external DNA segment related to 19 Chitinase genes from E.coli as the host; column1: size marker, column 2: extracted plasmid.

In this picture, the extracted plasmid DNA super coil lowest band, form a loose ring band above it and linear forms in the higher bands are visible.

External DNA segment tracking:

The size of the extracted plasmid on the gel is not a confident way to evaluate the existence of the segment inside the vector. So, PCR and enzymatic cutting were used.

Enzymatic cutting:

This method is one way to confirm the entry of the fragment into the vector and the recombinant plasmid cutting via restriction enzymes.

To do that, the recombinant plasmid was been cutting with NcoI enzyme, and after detection on the electrophoresis gel, we saw only one segment bigger than the empty vector, pGem-T (figure 5, 1th column). The plasmid containing the fragment of a point and a line has been cut. Once again, we did it with NcoI and SacI and there were two segments on the gel after electrophoresis. The size of one fragment was about the

wanted piece (750 – 1000 bp) and the other length was about the empty vector pGEM-T (about 3000 bp) (figure 5, 2th column). These observations were the cause of this fragment entrance into the plasmid.

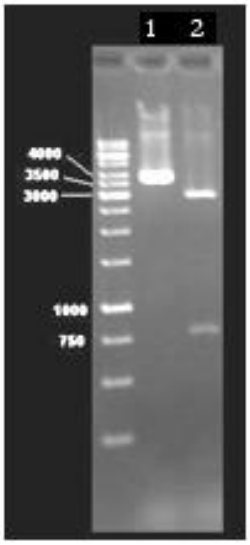


Figure 5:pGEM-T/chi9-gene, recombinant plasmid, enzymatic cutting. Column 1: mono enzyme cutting by NcoI. Column2: bi-enzyme cutting by NcoI and SacI. Size marker 1Kb: left.

PCR:

After polymerase chain reaction and PCR product detection, a band about the wanted length was observed on the gel (figure 6).

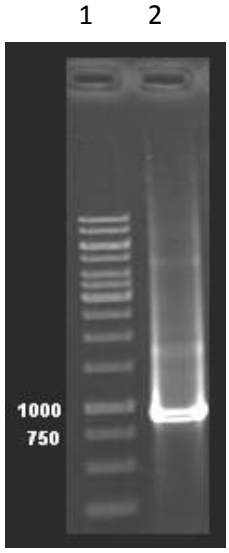


Figure 6: PCR product electrophoresis. Column 1: sixe marker 1Kb. Column 2: PCR product.

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