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Draft Genome Sequence of a Glutaminase production bacterium, Bacillus sp.B12, Isolated from Diyala Soil

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Abstract

Thirteen environmental isolate were isolated from soil from Diyala city, Iraq. The isolates were identified using phenotypic characteristic methods. All isolates were Gram-positive, spore-forming, motile, aerobic growth. The strians were tested for L-glutaminase production, apromising isolate, B12 presented the highest L-glutaminase activity. Taxonomic position for the selected bacterium (B12)was investigated on the basis of whole genome sequencing(WGS). Comparative computer database analyses revealed that the bacterium is Bacillus sp.B12 (Genbank accession number: SAMN12069785). In prediction of ***silico*** *for antimicrobial and metal-resistance of Bacillus sp. B12, by RAST tool.Many toxicity resistance and secondary metabolite genes were detected.*

In vitro*the selected isolate was optimized formaximum tolerance concentrations (MTC) for four metal salts: barium chloride ($BaCl_2$), cadmiumsulfate ($CdSO_4$), ferric sulfate ($FeSO_4$), nickel sulfate ($NiSO_4$), and zinc sulfate ($ZnSO_4$)in the 0-10 mM range.Bacillus sp.B12could tolerate 10mM of Ba^{2+}, and up to 6 mM of Zn^{2+}.*

The optimal inoculum size was 1.8×10^4 cell/ml that produced the highest L-glutaminase activity (4.22 U/ml).The enzyme maximum activity was accomplished 120u/min when the enzyme was incubated with 200 mM of glutamine at pH7.5 under 35ºC for 20 hrs. by using an incubation period of 20 hrs the maximal GLUase production wasachieved, with an optimum of pH 7.5, a temperature of 35 ºC for incubation, an agitation rate of 120 rpm. which produce a glutaminase and for further use of pharmaceutical use may be potential candidate.

Keywords:*Bacillus sp., Phenotypic characterization, WGS, L-glutamiace, metals tolerance .*

I. Background

The soil ecosystem is a complex network composed of interactions between thousands of organisms belonging to all three kingdoms of life: bacteria, archaea, and eukaryotes. Prokaryotes are mainly responsible for biological, geological and chemical processes in soil environments(Tecon and Or, 2017). Furthermore, by the

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quantity and quality of available substrates in an environment soil microbial biomass and abundance is regulated(Bardgett, 2005).

Microbial communities are an important biological part of soil function, and all organisms in an ecosystem rely on the activity of microorganisms for their role in improving soil quality and regulating nutrient availability (Nannipieri et al., 2003). Soil bacteria are the most abundant group within soil microbiota. For example, culture- dependent methods combined with molecular methods showed that the heterotrophic bacterial phyla *Bacteroidetes, Flavobacterium,* and the α-, β- and γ-*Proteobacteria* are the most predominant bacteria in soil environments (Ramirez et al., 2018).

The bacterial populations response to heavy metal contamination based on the concentration, bioavailability of the metal itself and microbial species. In heavy metal-polluted soils the microbial survival based on intrinsic biochemical properties, physical and genetic adaptation including environmental and morphological modifications (Abou-Shanab et al., 2007).

For many years characterizing microbial communities from the soil ecosystem has been largely restricted to culture methods (Rowe et al., 2017). Whole genome sequencing (WGS) is the most advanced molecular technique introduced to examine the phylogenetic association of bacteria. With the introduction of pyrosequencing and Illumina sequencing, WGS has become much more affordable, though various other platforms or technologies are available. A significant advantage of WGS over targeted gene sequence analyses is that it provides all the information content of DNA to isolate in a single rapid step following culturing of bacteria.

In bacterial genome sequencing whatever the technique applied, the general approach remains unaltered and contain, sequencing of DNA,sample preparation,assembly of sequence, and bioinformatics analysis (Abou-Shanab et al., 2007). Soil microbes generally rely upon passive transport through the soil matrix or use energy-dependent mechanisms of motility and chemotaxis.

They produce many of the extracellular enzymes which are needed to break down organic material for sustainable soil productivity (Abou-Shanab et al., 2007). In soil there are many examples of heavy metals such as mercury, cadmium, chromium, cobalt, copper, nickel and manganese (Zhang et al., 2015).

Metals such as copper, nickel , zinc, and chromium are important in the life of microorganisms. Others, for example mercury, cadmium and plumb, are non-essential (the role of biology is not known) (Hau et al., 2017).However, even the critical group of high-level metals may be harmful to microorganisms' growth,metabolism and morphology.Many microorganisms can tolerate existing metals in metal-polluted environments. No general mechanism for tolerance to toxic substances exists, there is plenty of evidence for tolerance for both chromosomal genes and plasmids.(Barba et al., 2014).

A largeassortment of soil microorganisms containing bacteria, yeast, mold, and filamentous fungi have been reported to produce L-glutminase. L-glutamine was especially interested in the anti-tumor behavior and usein the industry of food as an enhancer of flavor, as well as its use as a glutamine sensor without separate glutamine acid measurements (Tork et al., 2018).

Due to the limitations of anticancer activity of bacterialL-glutaminase enzyme, the present study aims to identify abacterial isolate based on phenotypic characteristic and whole genome sequencing, to investigate the optimum conditions to produce L-glutaminase.

II. Materials and Techniques

2.1 Chemicals

The culture chemicals and media used for production of enzymewere purchased from USA, Sigma-Aldrich Chemical Co. Nesseler's reagent was purchased from (Buchs,Switzerland)Fluka. The L-glutamine was obtained from Spectrochem (India).

2.2 Samples collection

Between March and January 2019, 20 soil samples were gathered from Diyala city in east of Iraq. The soil samples were collected in new polyethylene bags from different locations in Diyala city, Iraq. 1g of soil/ rotted tissue samples were transferred to a conical flask containing 99 ml of phosphate buffer saline (PBS) and tenfold dilution were made by using 9 ml PBS. From the desired dilution, 0.1ml of diluted sample was spread Plated on Nutrient Agar (NA) plates (provided with 5mg/L of cycloheximde), and incubated at 35ºC for 24 h. A total single distinct colony from all samples were picked up purified and kept in NA slants at 4°C until used for further studies.

2.2.1 Morphological and Phenological characterization

In present study, light microscopy investigated the morphology of obtained isolates. In addition phenotypic tests were completed:oxidase test, catalase, andmotility; methyl red test; Vogas-proskauer test, gas production from glucose; degradation of starch; acid from L-arabinose, D—xylose,D-glucose,sucrose and D-mannitol; nitrate reduction ; formation of indole; H2S production; DNase test (Labratories Pvt.Ltd,Mumbai).

2.3 Screening ofbacterial isolates for GLUase production

The bacterial isolates were screening for GLUase production using the rapid plate assay method (Rochelet et al., 2015),which was achieved using glutamine agar medium consisting of 0.3ml of 2.5%phenol red. After incubation at 35ºCfor two to three days, pink zone around the colonies was measured.Controls were three groups **a)** medium without dye, **b)** medium without L-glutamine ($NaNO_3$ instead, and **c)** non-inoculated medium.

2.4 Determination of L-glutaminase activity

The selected isolates (20 isolates) were grown in M9 medium and incubated on the rotary shaker as 150 rpm at 35ºC for 24h. A centrifugation at 8000 xg for 30 min was used to remove bacterial cell. The supernatant was used to assess the L-glutaminase production(Hammed and Alyaa, 2010).

2.5 The Determination of Protein

In determining the concentration of the enzyme, the Lowry et al (Peterson, 1977) approach was used. A standard protein stock solution, bovine serum albumine at a 1000 µg / mL concentration, was developed. Folin-

Ciocalteu reagent has been applied to each sample, with absorption estimated at 660 nm after incubation for 30 min.

2.6 Genome sequencing

The results of previous experiments clearly indicated that isolate *B12* has the maximium L-glutaminase production. Cultures ofB12were grown on nutrient agar for extraction DNA using Qiagen DNA mini kit (Qiagen,MD,United States) with the inferenceof a lysis step using lysozyme.

2.7 Annotation of de novo assembled genomes

To search for homology via gene calling and functional annotation, the contigs were exported as FASTA files and from this method, the number of contigs per genome was obtained.The subsystem technology (RAST) version 4.0 (http://rast.nmpdr.org), and SEED viewer program (Aziz et al., 2008) was applied to suspend the genes on each contig. In this process, version 11 of the genetic code was selected for bacteria. Then, genus, species and strain name was given, and finally, the organism's details were viewed under "Browse annotated genomes in SEED viewer".

2.8 Taxonomic affiliation

The assembled genomes in multi-contig format were used "at species level" and isolates were assigned "isolate number based on their soil origin". Since at this point the taxonomic identity of the species was unknown, the strains were given isolate numbers. To estimate the phylotypes of the selected genomes, the 16S rRNA gene sequences were retrieved from the RAST annotation and applied as query against the SILVA reference database with the threshold set to above 97%. To confirm the SILVA definition, the draft genome sequences were processed using the AmphoraNet tool to assign taxonomic affiliations of selected draft genomes (Kerepesi et al., 2014). This tool performs taxonomic classification by using 31 bacterial protein-encoding genes from the genomes under analysis, and if a putative taxon is assigned at least 75% of identified marker genes resulted in concordant taxonomy.

2.9Maximum tolerable concentration (MTC)

For the MTC of heavy metal salts, the chosen isolate was examined with minimal medium agar (MM agar), (Leedjärv et al., 2008)

. Stock solution of Ba^{2+}, Cd^{2+}, Zn^{2+},Fe^{2+}, Ni^{2+}, were used as $CdSO_4$,$BaCl_2$,$NiSO_4$, and $FeSO_4$ experimentally , the plates containing 20 ml above medium with various communication (2,4,6,8,10 mM). Bacterial strains were streaked on plates and incubated at $25^{o}C$ for 48- 72 hours. Finally,the isolate strain was checked for metal tolerance.

2.10 Optimization cultures conditions for L-glutaminase productions from of *Bacillus sp. B12*.

Optimal condition for the enzyme production were studied in order to obtain the highest yield.

2.10.1 Influence of inoculum size on L-glutaminase production

Different inoculum sizes ranged between 1.8×10^{1} and 1.8×10^{9} (cell/ml) were used to determine the influence ofinoculum size on production of L-glutaminase. Each experiment was performed in triplicate.

2.10.2 Effect of various incubation temperatures on L-glutaminase activity of *Bacillus sp. B12*.

The productivity level of L-glutaminase was determined after incubating the bacterial culture at various values of temperature that rating from 20 to 60 ºC for 24h.Each experiment was performed in triplicate.

2.10.3 Effect of various initial pH values on L-glutaminase activity of *Bacillus sp. B12*.

Activity of L-glutaminase in the production media was measured at various pH values to discuss the effect of pH on the production of the enzyme. The pH media was adjusted from 6 to 10, andcultures of bacterial were incubated at 35ºC for 24h. Each experiment was performed in triplicate.

2.10.4 Effect of various incubation periods on L-glutaminase activity of *Bacillus sp. B12*.

The effect of different periods of time incubation was determined after incubating the bacterial culture at 35 ºC from four to 48 h. Each experiment was performed in triplicate.

III. Results and Discussion

According to the physiological and morphological characteristic, all strains (13 isolate) belonged to the genus Bacillus sp. The biochemical and morphologicalproperties of the newly isolated strains are offered in Table 1. All strains demonstrated Gram-positive, spore forming, aerobic rod, motile and catalase positive. The strains can reduce nitrate, hydrolysis starch, production of H_2S, and gas production from glucose. Methyl red, Vogas-Proskauer, and DNase test activities listed in Table 1.All the morphological characteristics indicated that, these isolate belonged to Bacillus species. Many studies presented a diverse group of Bacillus species that obtained from the soil(Beneduzi et al., 2008). Some native isolates did not utilize carbohydrate for growth, and no glucose (Table 1).

Table 1: Characteristics of Phenological and Morphological for isolated strains from Diyla soil.

Characteristic	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**	**B9**	**B10**	**B11**	**B12**	**B13**
Cell shape	R	R	R	R	R	R	R	R	R	R	R	R	R
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+
Spore forming	+	+	+	+	+	+	+	+	+	+	+	+	+

Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+
Vogas-proskauer	-	-	-	-	-	-	-	-	-	-	-	-	-
Test of red Methyl	+	+	-	+	-	-	-	-	-	+	+	-	+
Production of gas from Glucose	-	-	-	+	-	-	-	-	-	+	-	-	-
Hydrolysis of Starch	+	-	-	-	-	-	-	-	-	+	-	+	-
Acid from D-glucose	+	+	-	-	-	-	+	-	-	-	-	-	-
Acid from D- xylose	-	-	-	-	-	-	-	-	-	-	-	-	-
Acid from L-arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-
Acid from D-mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-
Acid from Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-
Acid from Fructose	+	-	-	-	-	-	-	-	-	-	-	-	+
H_2S	+	-	-	-	-	+	-	-	-	+	-	+	+
Reduction of nitrate	+	+	-	+	-	+	+	+	+	-	+	+	+
Formation of indole	-	-	-	-	-	-	-	-	-	-	-	-	-
DNase	+	+	-	-	-	+	+	+	-	+	-	-	+

R: cell form rod; +:positive; -:negative

Theobtained isolates displayed L-glutaminase activity on M9-L-glutamine media at 35ºC for 24 h. The bacterium isolate B12 was the best isolate for the L-glutaminase generation by diving the largest pink zone diameter of 5 mm around the colony (Table2). Both results confirmed that the isolate B12 had the highest enzyme activity (3.5 U/ml).

Similarly, other study revealed that, morphological analyses of the bacterial colonies that isolated from the rhizosphere soil could provide a rich-source of L-glutaminase producing microorganism (Rosmin and Saramma, 2017). Selection of a suitable niche that yields potential producers of any metabolite is one of the most successful ways for assaying at suitable candidates for any target metabolites. There are many reports on L-glutaminase in soil. This is in contrast with Binod etal reported that most of the L-glutaminase production is from gram-positive bacteria (Binod et al., 2017).

Table 2: L-glutaminase activity in different bacterial isolates

Bacterial Isolates	Zone around colonies(mm)	Enzyme activity (U/ml)
B1	4.3	2.5
B2	4.2	2.7
B3	4.3	3
B4	4.5	2.2
B5	4.2	1.9
B6	4.5	2.0
B7	4.4	2
B8	3.9	2.2
B9	3.8	1.3
B10	4.3	2.4
B11	4.8	3.0
B12	5	3.5
B13	4.4	3.1

Sequencing of whole-genome of B12 isolate was achieved using Illumina MiSeq system. RAST based annotation predicted the number of proteins coding genes per genome to 5304 coding genes. Of these coding sequences, hypothetical proteins, devoid of any functional annotation, contributed with 5.5% of the total coding content per genome. The total number of rRNA gene sequences are 101 rRNA genes (Table 3).Genome feature characterization for each selected isolate showed similar properties in terms of genome size 4,981,595 and 39.9% GC content (with the range) to the closest genera in the NCBI database (Table 3).

Table 3: Summary of RAST output after annotation of *de novo* assembled genome of environmental *Bacillus* isolates.

Genome size	4,981,595
GC content	39.9

N50	29454
L50	44
Number of contigs	454
Number of coding genes	5304
Number of RNAs	101

The use ofannotation algorithmsand assembly software of *de novo* and is a strong approach that allows bacterial genomes to be assembled with automated annotation, which allow vast quantities of sequenceresults are converted into relevant datathat are important for public comparison of newbacterial strains (Lettieri and Kisand , 2013). WGS assembly and annotation aids the description of core genomes and various features of metabolic of new environmental bacteria, e.g. size of genome and fundamental pathways of metabolic.

3.1 RAST annotation

By analysis of RAST, the annotated ORFs of *Bacillus* were categories as reported in Figure 1. As a classification's result, in B12, the representing of subsystem categories to the metabolism of amino acids, carbohydrates,vitamins,cofactors, and pigments or prosthetic groups. A total of 168 ORFs are consist of in metabolism fatty acids, isoprenoids and lipids, while 33ORFs take part in metabolism of aromatic compounds. A total of 626 amino acids including glutamine dehydrogenase (10%) and rest annotated genes are predicted to involved in many metabolism pathways.And 64(2%) antibiotics –resistance genes such as beta-lactamase related proteins,penicillin-binding proteins, drug/metabolites transporters(Fig.1).

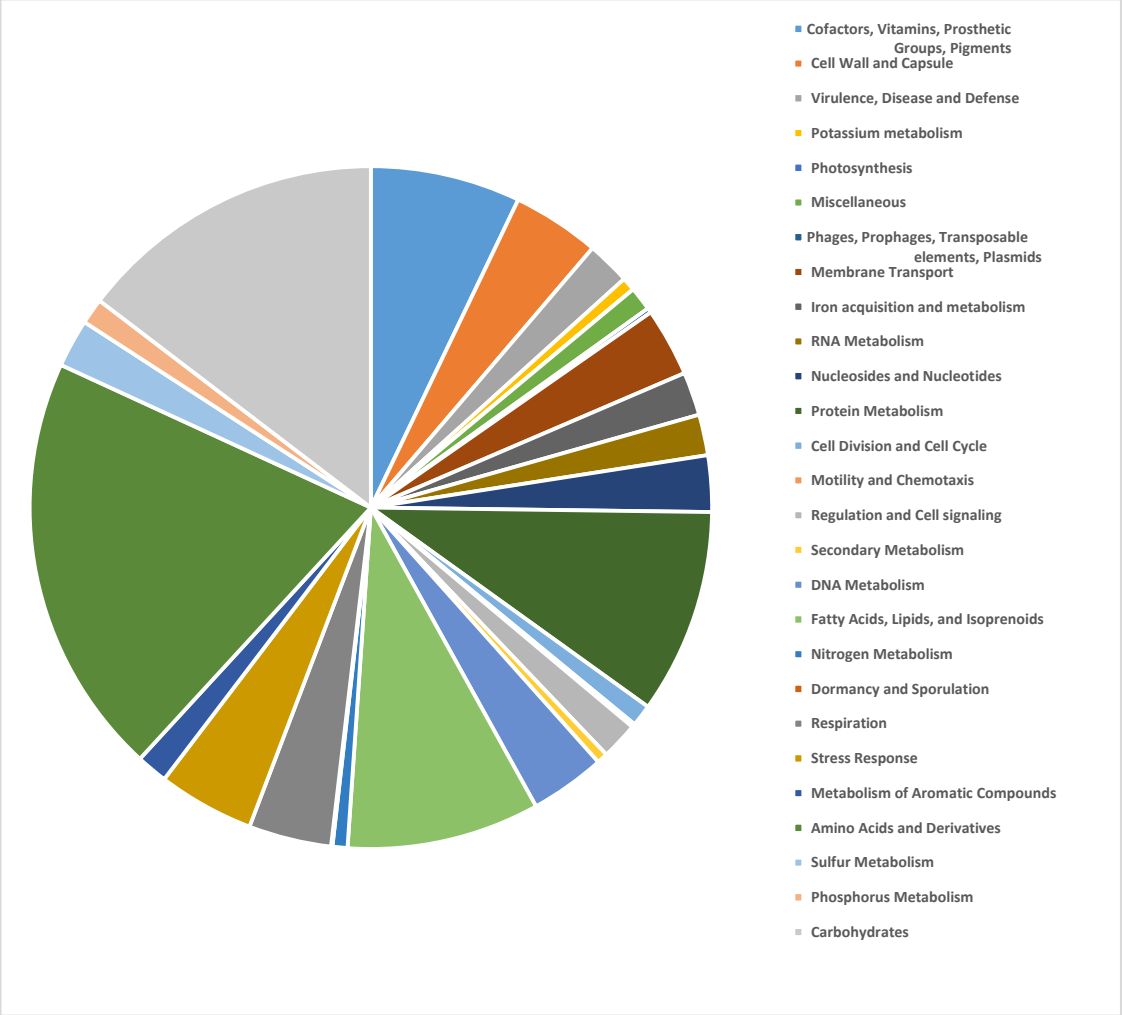

Fig 1:Number of the genes with associated functional categories of *denovo* assembled environmental *bacillus* genome. Overview of the total number of predicted functional proteins among the conserved region in all draft genomes (with exception *S. griseus* NBRC 13350). The functional annotations were given for the predicted coding sequences to identify the genes. The legend displays the highest ratios of putative protein functions.

3.1.1 Identification and characterization of putative antibiotic resistance genes (ARGs) with RAST/SEED

The number of predicted antibiotics and metalresistance genes was obtained by browsing the annotated genomes in SEED viewer based on homology to genes in the RAST database. The functional sub-categories system pointed to similar number of resistance genes in all genomes (Table 4). There are five genes that encode putative beta-lactamase enzymes, and four gene markers of fluoroquinolone resistance genes. Only one predicted gene encodes resistance to vancomycin (VanW), and only one gene marker was detected for Fosfomycin resistance, other genes encoding multi-drug resistance and Multidrug Resistance Efflux Pumps for several antibiotic classes such as MacB and Acriflavin resistance protein.

The subsystem feature toxic compounds resistance displayed four predicted genes that putatively encode for arsenic resistance, whereas three predicted genes encoded resistance to cobalt-zinc-cadmium and only

five predicted genes enable copper homeostasis. For bile hydrolysis, two putative genes were detected, and finally only two genes are putatively encoding a zinc resistance. An earlier study demonstrated that the microorganisms isolated from coal combustion ash settling basins were resistant to Ni^{2+}, and displayed resistance to 30 mg/L of ciprofloxacin (Van Nostrand et al., 2007) found that many efflux pump systems contribute to antibiotics resistance in *Acinetobacter baumanii* strains (Lin and Lan, 2014).

Table 4: The total number of antibiotic and toxic compounds resistance genes was obtained from the annotated genomes of *Bacillus* to the SEED subsystem and computing the number of predicted genes under functional Sub-categories / virulence, disease and defense window. Functional categories of putative genes contained within contigs unique to isolates and not present in reference genome. [a] The highest first 20 putative proteins that predicted by RAST annotation tool.

Role	Features
Copper homeostasis	Multicopper oxidase
Copper homeostasis	Copper-translocating P-type ATPase (EC 3.6.3.4)
Copper homeostasis	Copper resistance protein CopC
Copper homeostasis	Multidrug resistance transporter
Copper homeostasis	Copper resistance protein D
Bile hydrolysis	Alpha-ketoglutarate-dependent taurine dioxygenase (EC 1.14.11.17)
Bile hydrolysis	Choloylglycine hydrolase (EC 3.5.1.24)
Resistance of cobalt-cadmium- zinc	Resistance protein of cobalt-zinc-cadmium
Resistance of Cobalt-zinc-cadmium	Resistance protein CzcD of Cobalt-zinc-cadmium
Cobalt-zinc-cadmium resistance	Transcriptional regulator
Vancomycin Resistance	Vancomycin B-type resistance protein VanW
Resistance of Zinc	Resistance of Zinc -associated protein
Resistance of Zinc	Regulator response of zinc sigma-54-dependent two-component system

Resistance of Multidrug	2-protein version Found in Gram-positive bacteria
Resistance of Multidrug	2-protein version Found in Gram-positive bacteria
Resistance of Multidrug	2-protein version Found in Gram-positive bacteria
Fluoroquinolones Resistance	DNA gyrase subunit B (EC 5.99.1.3)
Fluoroquinolones Resistance	DNA gyrase subunit A (EC 5.99.1.3)
Fluoroquinolones Resistance	Topoisomerase IV subunit B (EC 5.99.1.-)
Fluoroquinolones Resistance	Topoisomerase IV subunit A (EC 5.99.1.-)
Resistance of Arsenic	Arsenical resistance operon repressor
Resistance of Arsenic	Arsenic efflux pump protein
Resistance of Arsenic	Arsenate reductase (EC 1.20.4.1)
Resistance of Arsenic	Anion permease ArsB/NhaD-like
Resistance of Fosfomycin	Fosfomycin resistance protein FosB
Lactamase-Beta	Beta-lactamase class A
Lactamase-Beta	Beta-lactamase class C and other penicillin binding proteins
Lactamase-Beta	Beta-lactamase (EC 3.5.2.6)
Lactamase-Beta	Beta-lactamase repressor BlaI
Resistance of Cadmium	Cadmium efflux system accessory protein
Efflux pumps multi-drug resistance	Multi antimicrobial extrusion protein (Na(+)/drug antiporter)
Efflux pumps multi-drug resistance	Macrolide export ATP-binding/permease protein MacB (EC 3.6.3.-)

Efflux pumps multi-drug resistance	Acriflavin resistance protein
Efflux pumps multi-drug resistance	Multidrug-efflux transporter

3.2 Taxonomic identification

Taxonomic classification depend on 16S rRNA gene sequence analysis using the SILVA database as reference revealed that the sequenced isolate(B12) belonged to the genus *Bacilluslicheniformis*.Similarly,the AmphoraNet tool, using 31phylogenetic marker genes, provided a more detailed classification confirmed that the isolate B12 belong to bacillus sp.and a taxon name was allocated if at least 75% of specified marker genes resulted in putative taxon and concordant taxonomy (Table 5).

Taxonomic classification of the assembled draft genomes placed the selected isolate into *Firmicutes*. Earlier studies of bacterial communities associated with urban soils demonstrated that, the predominant bacterial genera belonged to the *Firmicutesm*(Reber et al., 2016). The observed variation in resistance to antibiotics among these culturable bacterial isolates from different soil samples may reflect a history of exposure to specific metals (Zhang et al., 2013).

Table 5: Phylogenetic affiliation of bacterial isolate obtained from soil samples.

Isolates ID	SILVA[a]	AmphoraNet [b]
Sample 12	*Bacillus*	*Bacillus licheniformis*

[a] Taxanomic classification of the 16S rRNAgenes at similarity threshold of 97 %.

[b] The best homology matches with 31 marker genes (conserved housekeeping protein-encoding genes) detected by AmphoraNet.

3.3 Maximum tolerable concentration (MTC)

The quality of the growth of isolates was presented and scored in Table 4. The selected isolate couldafford high concentricity of Ba^{2+}(10mM). while it showed maximum tolerance to $ZnSO_4$at 6mM (Table 6).However it lose the capacity to grow on Cd^{2+}, Fe^{2+} and Ni^{2+}medium.

MTC was specified as the highest concentration of metal salt that still allowed growth after 72 hr (Schmidt and Schlegel, 1994). Bacterial strains have ‘intrinsic’ activity of broad-spectrum efflux pumps systems which are mediated by several genes (D'costa et al., 2006).

The environmental *B. mycoides* isolates were classified as resistant to ciprofloxacin, tetracycline and

vancomycin, in contrast to several *B. mycoides* isolates from culture collections which were sensitive (Luna et al., 2007).Nickel- resistant *Bacillus megaterium* SR28C, which was isolated from metal-contaminated soil displayed resistance to tetracycline at 30µg/ml, and many other clinically relevant antibiotics (Rajkumar et al., 2013).

Table 6: Maximum Tolerance Concentration (MTC) of metal salts for *Bacillus sp.* MTC values were known as the highest concentration of metal that did not affect bacterial growth.

Metal salts	Concentration (mM)				
	2	4	6	8	10
$BaCl_2$	+	+	+	+	+
$CdSO_4$	-	-	-	-	-
$FeSO_4$	-	-	-	-	-
$NiSO_4$	-	-	-	-	-
$ZnSO_4$	+	+	+	-	-

- Growth not visible withintwo days; + Growth visible within two days.

3.5 Optimization of culture conditions for L-glutaminase production

3.5 .1Influence of inoculum size

The inoculum size had potential role in the synthesis of L-glutaminase by B12. There was a gradual increase in the production of enzyme until reaching the greatest activity (4.2 U/ml) by the best inoculum size of 2 ml (containing 1.8×10^4cell/ml) under the experimental conditions (Fig.2).

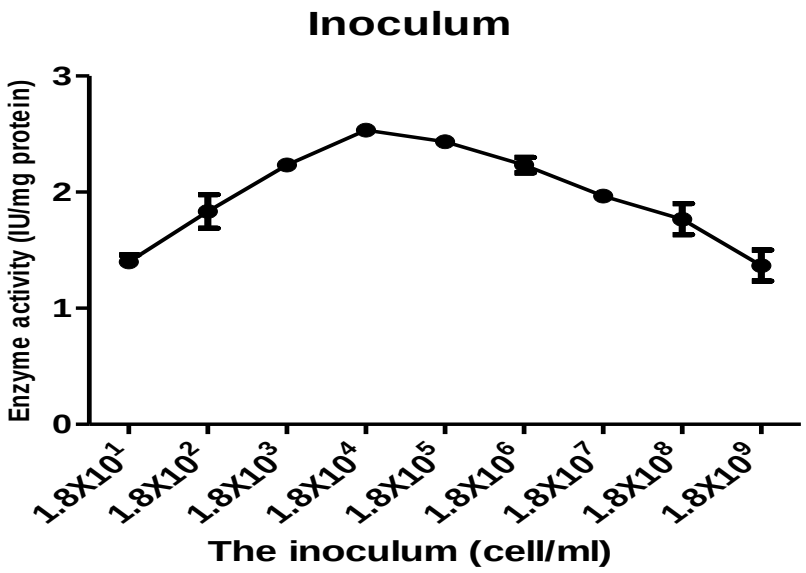

Figure 2: Influence of inoculum amount on L-glutaminase production.

3.5 .2 Effect of Temperature onL-glutaminase production

L-glutaminase activity, using various temperatures rating from 20 to 60 ºC (Fig.3). The results showed high activity at 35ºC, and the enzyme activity was 2.03 IU/mg protein. The enzyme production decreased gradually with increase the temperature to become 0.2 IU/mg protein at 60 ºC. The optimum temperature value that obtained from these assay was used in all other experiments. The effectiveness of temperature on production of L-glutaminase is linked to the growth of microbe.Optimum temperature for L-glutaminase activity was found to be the rating between 25 and 35 °C for the mesophilic bacteria isolated from environment (Wallenstein et al., 2010). The current study recorded 35 °C as optimal, which agrees with earlier finding (Krishnakumar et al., 2011).

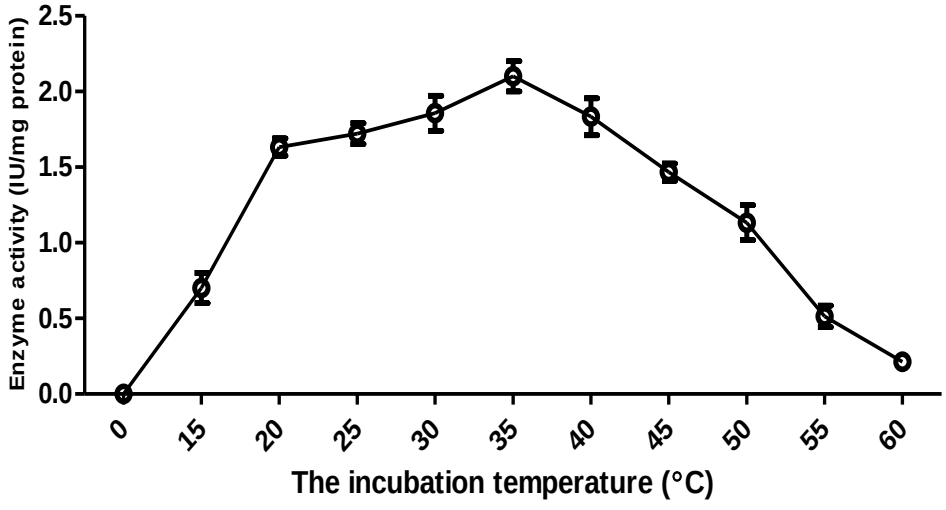

Figure 3: Influence of temperature on production of L-glutaminase.

3.5 .3 Effect of incubation time on production of L-glutaminase

In order to determine theinfluence of incubation time on L-glutaminase production from E.13, the enzyme was carried out by incubating the selected isolate on the production media at different time periods. The enzyme activity was determined after four hours of an incubation, and the enzyme activity was 0.5 IU/mg protein. As shown in figure 3, the maximum production of enzyme was after 20 hr. of incubation and the enzyme activity was 1.8 IU/mg protein. However, the activity was dropped gradually to become 0.3 IU/mg protein after 48hr of incubation (Fig.4). The incubation period varies with enzyme productions. The maximum L-glutaminase yield recorded with *Bacillus B12* is an appreciable level when compared to earlier reports for environmental bacteria. The L-glutamnase production increased during the logarithmic phase followed by decline on extended incubation (Meena et al., 2015),this also true for the present study.

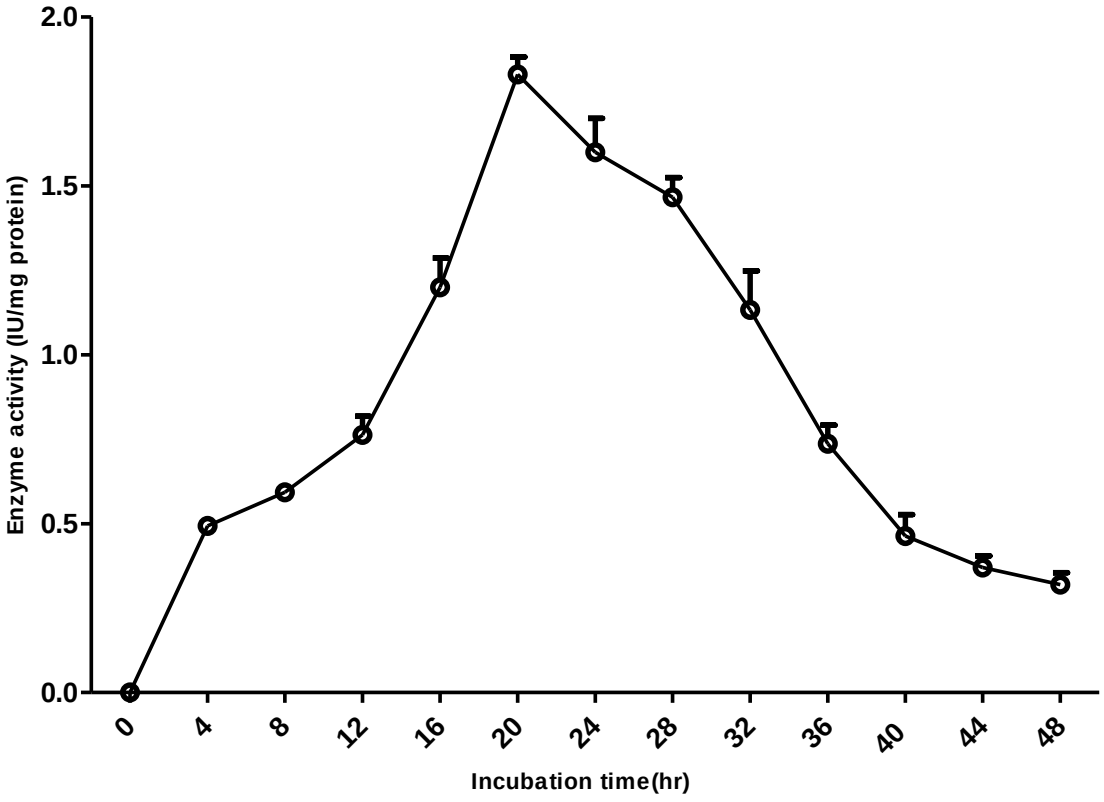

Figure 4: Influence of incubation time on production of L-glutaminase.

3.5.4 Effect of pH on production of L-glutaminase

The optimum pH value of L-glutaminase enzyme from *Bacillus*B12 was determined for the pH range from 6.0-10. The highest enzyme production was determined at pH 7.5, the enzyme activity reached 2.5 IU/mg protein. Whereas the activity of enzyme was dramatically reduced to become 0.2 IU/mg protein at pH 10 (Fig.5). Among physical factorsstudied, pH of the medium plays an useful role by inducing changes of morphological in microbes and enzymes secretion. Neutral pH is required for maximal extracellular enzyme activity of

Aeromonas veronii(Ray et al., 2012). This is true for *Bacillus B12* used in this study.To conclude, isolation and screening of enzyme producing bacteria from environment may scope to access their therapeutic potential.

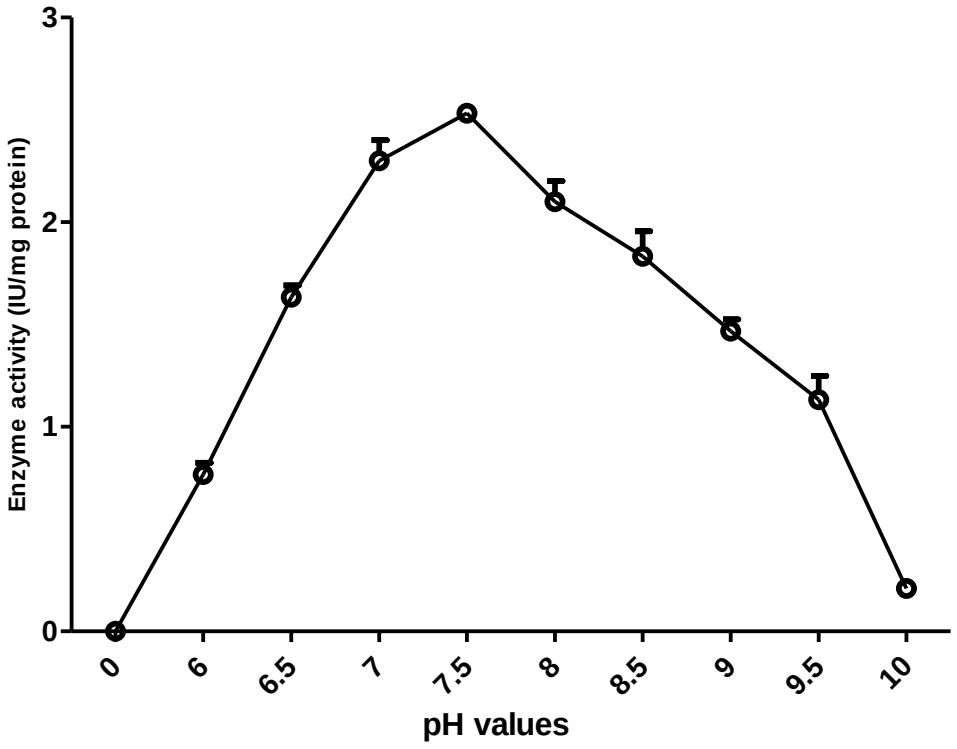

Figure 5: Influence of pH values on production of L-glutaminase

Conflict of interest

In relation to the publication of this article, the writers assert no conflict of interest.

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Supplementary materials

S1: the whole genome sequence (fasta file)

S2: AmphoraNet output

S3: RAST output (subsystem categories)

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