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Study on the Production of Vitamin B_{12} by Newly Isolated and Identified *Pseudomonas aeruginosa* and Effect of Gamma Irradiation on Fermentation Substrate

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ABSTRACT

Vitamins are organic compounds that are not produced by body cells. Therefore, vitamins need to be taken from nutrients and supplements daily. They play an important role in carbohydrate, fat and protein metabolism, healthy development of the body, immunity against infections, and digestive functions. Vitamin B_{12} is a necessary nutrient; it is widely used as a dietary supplement and to treat anemia all over the world. The present study focuses on isolation and extraction of vitamin B_{12} producing *Pseudomonas aeruginosa* isolated from bladder cancer patients urine samples, the isolate was identified using morphological, physiological, biochemical methods, and confirmed by API 20 specific media, and named *Pseudomonas aeruginosa* M20. The vitamin B_{12} production was enhanced by using Trypticase soy broth and vitamin B_{12} like substance was extracted by using methanol –water as solvent. More over Two doses of Gamma irradiation were used (1 and 2 kGy) to study its effect on vitamin B_{12} like substance production. The extracted vitamin B 12 like substance was characterized and identified by using HPLC, IR and EDX compared to commercial vitamin B_{12}, different media (Mineral salt - methanol medium and Trypticase soy broth medium) were used for Vitamin B_{12} production. In addition, different solvents (methanol –water and acetone) were used for extraction of vitamin B_{12}. Trypticase soy broth and methanol –water were the most proper medium and solvent for Vitamin B_{12} production with compared by commercial vitamin B_{12} standard. The results presented in HPLC graphs exhibits a peak with RT at 5.422 of standard vitamin B_{12}, while on Trypticase soy broth medium and methanol-water as a solvent showing a peak with RT at 5.522 which is closely related to standard Vitamin B_{12}. The antimicrobial activities of both irradiated and non-irradiated bacterial extracts against bacterial and fungal strains were tested and two doses of Gamma irradiation were used (1 and 2 kGy) to study the effect of Gamma irradiation on the anti-microbial activity of the extract against the bacterial and fungal strains.

INTRODUCTION

Pseudomonas aeruginosa is pathogenic bacteria involved in numerous diseases among which, are urinary tract infections (UTIs). The pyocyanin pigment secreted as a virulence factor by this bacterium has many beneficial applications, but its high cost remains an obstacle for its widespread use [1]

It's considering an opportunistic pathogen that causes severe chronic infections in immune-compromised patients and other, such as cystic fibrosis (CF) or chronic obstructive pulmonary disease (COPD) patients. The key to *P. aeruginosa* survival in environments and hosts that range from soil to various living host organisms is its metabolic versatility. It subsists on various carbon sources for energy and the uses nitrogen as a terminal electron acceptor under anaerobic conditions, requires minimal nutrients, and grows at temperatures up to 42°C. Also, *P. aeruginosa* uses anaerobic metabolism process to reduce nitrogen (N2) via the denitrification as an essential metabolic condition during chronic infection and biofilm [2]. During the infection of P. aeruginosa , the bacteria must multiply inside the infected organisms (plant, animal, insect, etc.), requiring active DNA synthesis for bacterial cell division [3].

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Vitamin B_{12} is considered as one of the most structurally complex cofactors synthesized by bacteria, however, not all microorganisms encode for the ~25 genes needed for the complete biosynthetic pathway. In nature, two pathways of vitamin B_{12} biosynthesis exist: the aerobic or late cobalt insertion pathway and the anaerobic or early cobalt insertion pathway. One of the genes involved in the aerobic pathway that participates in cobalt insertion is the cobN gene described extensively in *Pseudomonas denitrificans*.[4]

Pseudomonas synthesizes vitamin B_{12} for different metabolic reactions, such as methionine synthesis, cobalamin biosynthesis, and RNR enzymes. One essential reaction is ribonucleotide synthesis by RNR. *P. aeruginosa* PAO1 has been demonstrated to grow in filament cell morphology due to cellular stress by RNR activity depletion, such as the low expression levels of class III RNR under anaerobic conditions. [5]

Vitamin B_{12} is a water soluble and one of the smallest, but structurally complex molecules widely recognized in the field of biological sciences that functions as a cofactor essential in many biological fields, as fat biochemical reactions, protein metabolism and hemoglobin synthesis as well as DNA synthesis and regulation [6]. Furthermore, vitamin B_{12}, also called cobalamin, has a key role in the normal functioning of the brain and nervous system and the formation of red blood cells (RBCs). It is one of eight B complex vitamins groups [7]. Vitamin B_{12} exhibited a class of chemically related compounds (vitamer) which exhibits a pharmacological activity. It contains the biochemically rare element cobalt (chemical symbol Co), which is positioned in the center of a planar tetra-pyrrole ring and called a corrin ring. Vitamin B_{12} is also produced by bacteria in the form of hydroxocobalamin, but conversion between different forms of the vitamin occurs in the body after consumption. [7]

Neither The fungi, plants, nor animals are capable of producing vitamin B_{12}.The bacteria and *archaeabacteria* only have the enzymes needed for its synthesis. Proved food sources of B_{12} are animal products (meat, fish, and dairy products). Some researchers reported that certain non-animal products can possibly be a natural source of B_{12} because of their bacterial symbiosis. It is also noticed that vitamin B_{12} is the largest and most structurally complicated vitamin which can be produced industrially only through a bacterial fermentation-synthesis. The synthetic of Vitamin B_{12} also used to fortify foods as a dietary supplement. [8]

Other Microorganisms used for production Vitamin B_{12} in the industrial field is achieved through fermentation of selected microorganisms such as the *Streptomyces griseus*, which was the commercial source of vitamin B_{12} for many years. Moreover, the *Pseudomonas denitrificans* and *Propionibacterium freudenreichii subsp. shermanii* are more commonly used today, and they are frequently cultured at special growth conditions to enhance yield. [9]

The Pseudomonas aeruginosa is a Gram- negative, rod-shaped bacterium, which causes disease in animals and humans. The microorganism is considered opportunistic insofar as serious infection which is often super imposed upon acute or chronic morbidity – most notably cystic fibrosis and traumatic burns – or found in immuno compromised individuals. [10]

Pseudomonas aeruginosa is a producer of many enzymes as citrate, catalase and oxidase , which habitats in many environments as soil, water, skin flora, and most man-made activities throughout the world. *Pseudomonas aeruginosa* also consider a facultative anaerobe, as it is well adapted to proliferate in conditions of partial or total oxygen depletion, which can achieve anaerobic growth with nitrate or nitrite as a terminal electron acceptor. Also when oxygen, nitrate and nitrite are absent, it is able to ferment arginine and pyruvate by substrate-level phosphorylation process. [11]

Therefore the present study aimed for the production, isolation and identification of B_{12} by *Pseudomonas* sp isolate and evaluate gamma irradiation effect on vitamin B_{12} production.

MATERIALS AND METHODS

-Isolation and purification of bacterial cells from clinical sample

In the current study, a total of fifteen urine samples used for the isolation of *P. aeruginosa* were collected from patients suffering from bladder cancer. The urine samples were centrifuged at 7000 rpm for 5 minutes in sterile falcon tubes, the sediment was inoculated on nutrient agar medium. Yeast extract 2.0, Lab lemco powder 1.0, Peptone 5.0, NaCl 5.0, Agar 15.0, the composition in g/L were dissolved in distilled water up to 1000 ml. and the pH was adjusted to 7.0 - 7.4. Pseudomonas agar medium was used (for fluorescein production). It has the following composition: (g/L) (Proteose peptone 20.0, Tryptone 10.0, Dipotassium phosphate 1.5, Magnesium sulfate 0.73, Glycerol (Difco)

10.0ml, Agar 15 g). These were dissolved in distilled water up to 1000 ml, pH was adjusted at 7.1). The medium powder and glycerol were added to water; the mixture was heated to boiling to dissolve ingredients. The blue-green pyocyanin pigment is a well-known phenazine pigment produced by about 95% of *P. aeruginosa* strains. It is considered a biomarker for identification of *P. aeruginosa*. Seven genes are involved in the biosynthetic pathway of pyocyanin, but phzM and phzS are the principle ones in conversion of phenazine-1-carboxylic acid to pyocyanin. [12]

The samples were collected from El-Noor Lab, Cairo, Egypt. The samples were cultured and incubated in proper conditions. The single separated colonies were picked up and Purified by several subcultures. [13]

-Characterization of bacterial isolate using API media

These methods were recommended by Bergy,s manual of determinative bacteriology ninth edition 1994 which were used for the characterization of the bacterial isolates. The characterization was assessed by API media 20.

- Characterization by Scanning Electron Microscope

Scanning Electron Microscopy: ZIESS, EVO 15, UK at the National Center for Radiation Research and Technology (NCRRT), Cairo, Egypt was utilized.

The basic principles of isolation, fixation, dehydration, drying, mounting, and photographing have many variations, scanning electron microscopy can be used for viewing microorganisms under this study; however, the concentration of cells is critical. [14]

-Catalase test: (3% hydrogen peroxide) and **Coagulase Test**: slide method was carried out according to the method recommended by. [15]

- Oxidase test: Filter paper test was carried out according to the method recommended by. [16].

-Extraction and purification of bacterial metabolites

The bacterial strain was inoculated on different media (Mineral salt-Methanol medium and Trypticase soy broth medium) and incubated at 37°C for 7 days. The broth was then filtrated through filter paper (Whatman no1) followed by centrifugation at 5000 rpm for 10 min. The clear filtrate containing the active metabolite was adjusted at pH 7.0 then the extraction process was carried out using different solvents (Methanol –water and acetone) at the level of 1:1 (v/v). The organic phase was collected and evaporated under reduced pressure using rotary evaporator. [17]

-Effect of Gamma irradiation on Vitamin B_{12} production and its antagonistic properties

The dried bacterial suspension were irradiated at dose levels (1.0 and 2.0 kGy) to study the effec of γ-irradiation on the production of Vitamin B_{12} -like_substances dried bacterial cells was irradiated in the Co^{60} facility at the γ-irradiation unit (4000 A), NCRRT (At Russian unit, the dose rate at the time of experiment was 1.854 kGy)/hr. This method was recommended by [18].

-Antagonistic properties of the bacterial isolate

The antimicrobial activities of the bacterial extract were tested using the classical diffusion methods. Generally, this method depends on measuring the inhibition zones of the microbial growth in millimeter. The plates were incubated at 37°C for 18 - 24 hr. for pathogenic bacteria and at 28°C for 48 hr. for Fungi.[19]

-Determination of physiochemical properties of bacterial extract

-Solubility

The bacterial extract was tested for solubility with dimethyl sulphoxide **(**DEMSO**),** distilled water, acetone, Methanol, Ethanol and ethyl acetate.

-Spectroscopic analysis For Vitamin B_{12} like substance

-Infrared (IR) spectra were recorded using potassium bromide discs technique on FT-JASCO IR/6300 Spectroscopic apparatus (NCRRT, Cairo, Egypt).

- HPLC spectra recorded the bacterial extract which dissolved in the mobile phase (Acetonitrile and water 60 – 40 respectively) at a wave length of 250 nm, UV detector, temperature at 35^0C, flow rate (1ml/min.) and injection volume (5 ul) Colum was used **(Agilent, Eclipse XDB.CB, PN993967.906, SN USRKD 12619, 5um and 4.6 x 150 mm)** using **Agilent** 1100 series (NRC), Cairo, Egypt).

- **EDX** Spectra recorded the bacterial extract which was dried, picked up by spatula and separated on stub for SEM-EDX investigation, ZIESS EVO 15, Germen (NCRRT, Cairo, Egypt).

RESULTS AND DESICCATION

P. aeruginosa is a highly resistant pathogenic bacterium that accounts for several infections among which, is the urinary tract infection. The ability of most *P. aeruginosa* strains to form biofilms and adhere to urinary catheters and urothelium makes urinary catheterized patients at a high risk for developing UTIs. Most of research studies have been focused on the effect of pyocyanin, a secondary metabolite secreted by *P. aeruginosa*; on human respiratory system. Lately, more studies were directed to explain pyocyanin effect on different human body systems with special concern to the urinary tract. [20]

[21] Reported that urinary diversion is performed on a regular basis in urological practice. Surgeons tend to underestimate the metabolic effects of any type of diversion. From the patient's perspective, diarrhea is the most bothersome complaint after urinary diversion. This might be accompanied by malabsorption syndromes, such as vitamin B_{12} deficiency.

In the present study, fifteen urinary samples were used for isolation of *P.aeruginosa* strains, one strain was selected. The results shown in Tables (1& 2) and Figure (1) exhibited morphological and physiological characters of the clinical bacterial isolate, which was Gram negative, motile rods in shape, and it has the ability to hydrolyze the starch producing catalase and oxidase enzyme. It also exhibited positive result toward the beta- hemolysis on blood agar medium. The isolate was characterized by the following classical tests according to Bergey's Manual of determinative bacteriology ninth edition, 1994, and it was found to be *Pseudomonas aeruginosa* and called *Pseudomonas aerugiinosa* M20.

Solubility of Vitamin B_{12} -like substance

The extract was soluble in distilled water, ethyle acetate, ethanol and methanol, while it was non soluble in DEMSO.

Table (1): Carbohydrate fermentation of bacterial isolate

Test parameters	Test results
Glucose fermentation	+ ve
Galactoe fermentation	+ ve
Rahmenoe fermentation	- ve
Raffinoe fermentation	- ve

Table (2): Physiological and biochemical properties of bacterial isolate

Test parameters	Test results
Nitrate(NO_3) reduction	+ ve
Indol production	- ve
Argenine dihydrolase	- ve
Urase	+ ve
Esculin hydrolysis	+ ve
Gelatin hydrolysis	+ ve
Beta-glucosidase	- ve
D-glucose assimilation	+ ve
L-arabinose assimilation	+ ve
Mannose assimilation	+ ve
Manitol assimilation	+ ve
N-Acetyle-glucosamine assimilation	+ ve
D-maltose assimilation	- ve
Potassium gluconate assimilation	+ ve
Capric acid assimilation	+ ve
Adipic assimilation	+ ve
Malic assimilation	+ ve
Trisodium citrate assimilation	+ ve
Phenylacetic acid assimilation	- ve
H_2S production	- ve

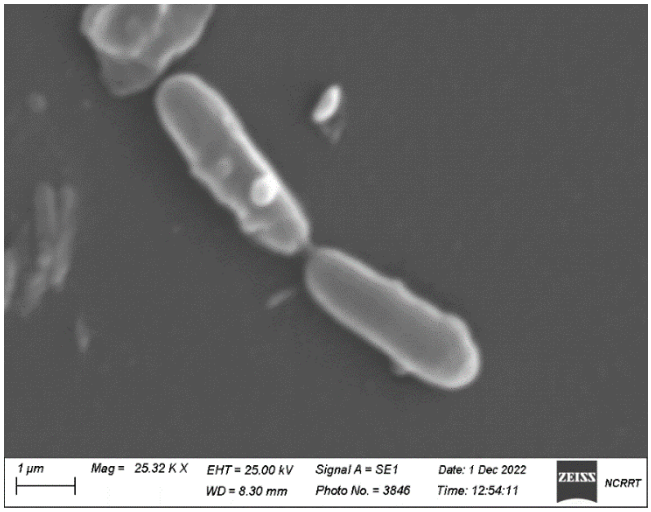


Fig. (1): SEM of *Pseudomonas aeruginosa* M20

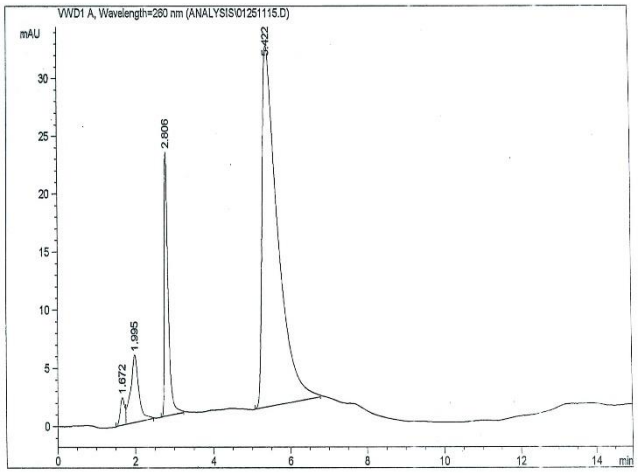


Fig. (2): HPLC chromatogram of vitamin B_{12} at peak 5.422

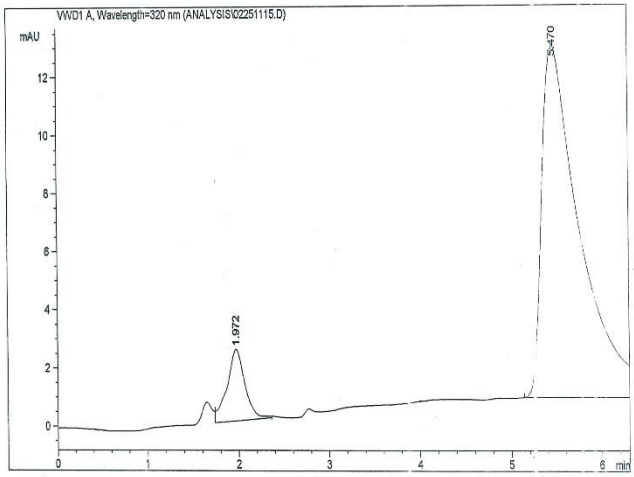


Fig. (3): HPLC chromatogram of extracted B_{12} at peak 5.622 (Trypticase soy broth medium and methanol-water solvent)

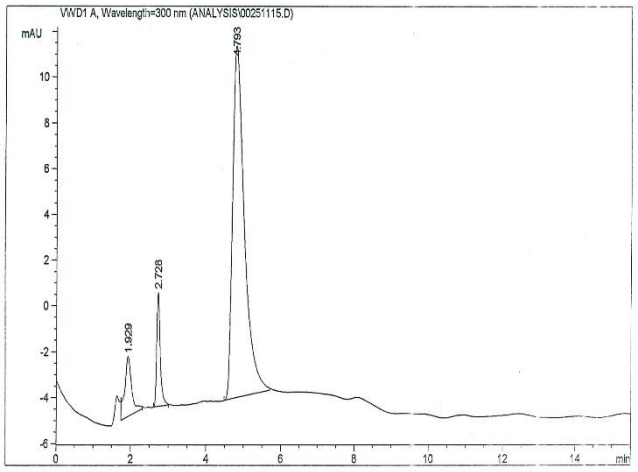


Fig. (4): HPLC chromatogram of extracted B_{12} (Trypticase soy broth medium and acetone solvent)

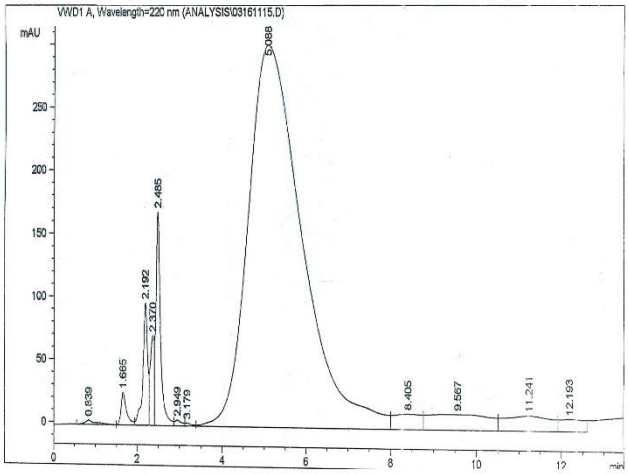


Fig. (5): HPLC chromatogram of Vitamin B_{12} like substance on Mineral salt methanol medium and acetone

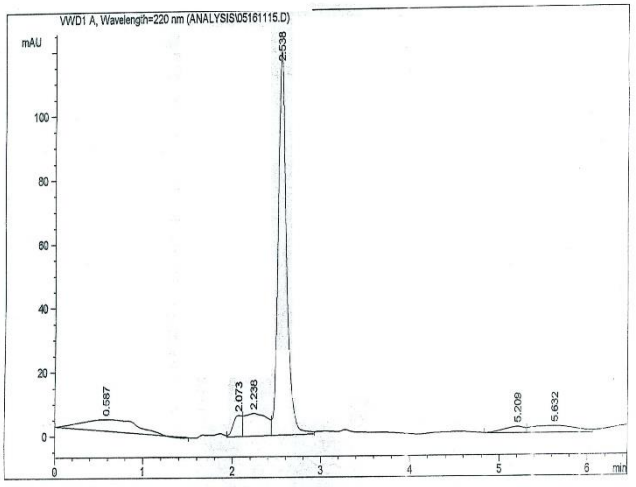


Fig. (6): HPLC chromatogram of Vitamin B_{12} like substance Mineral salt methanol medium and methanol-water

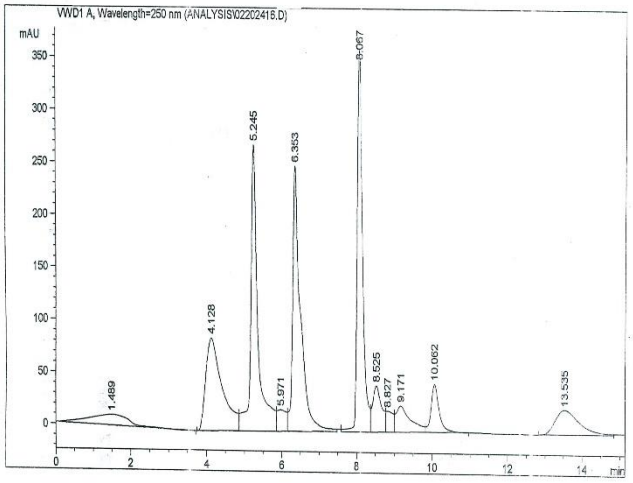


Fig. (7): HPLC chromatogram of Vitamin B_{12} like substance on Trypticase Soy broth media and acetone after Gamma irradiation at 1.0 k Gry

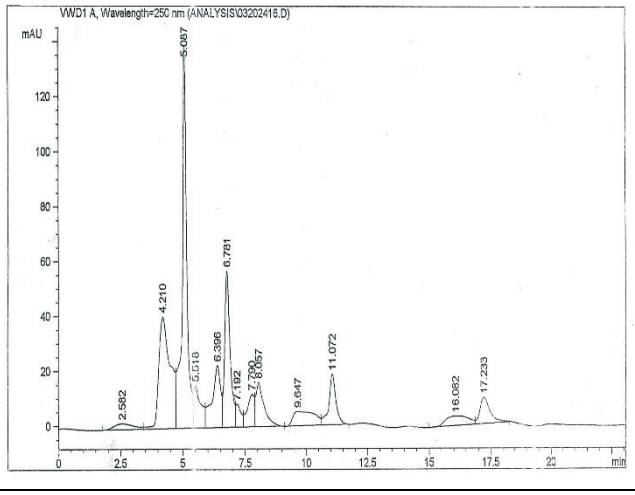
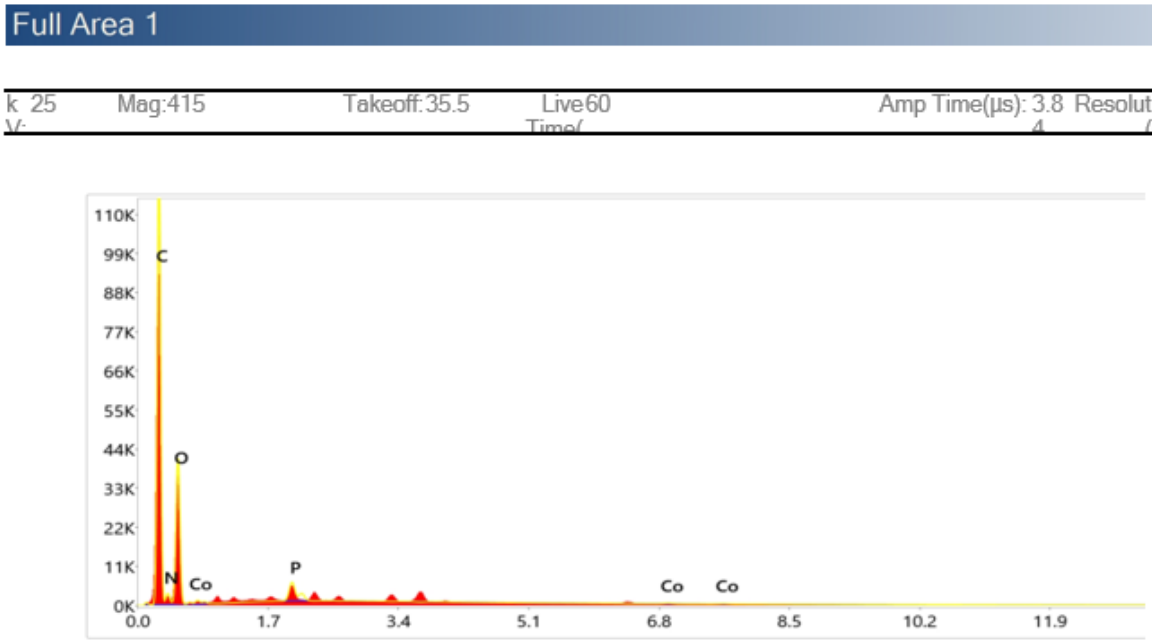


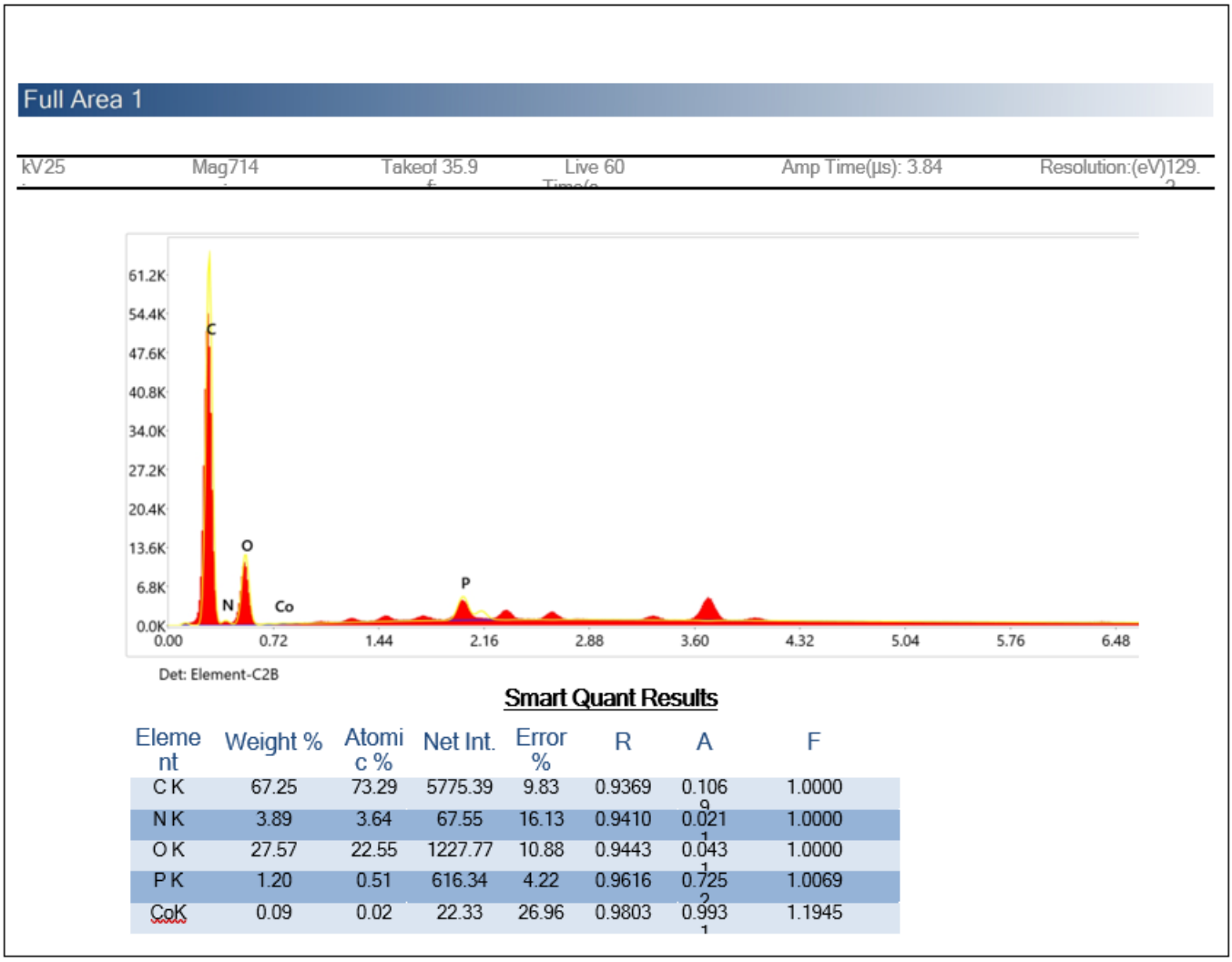
Fig. (8): HPLC chromatogram of Vitamin B_{12} like substance on Trypticase Soy broth media and acetone after Gamma irradiation at 2.0 k Gry



Smart Quant Results

Element	Weight %	Atomic %	Net Int.	Error %	R	A	F
C K	57.67	64.26	11180.7	9.83	0.9356	0.1033	1.00
N K	5.51	5.26	258.19	14.03	0.9398	0.0244	1.00
O K	36.06	30.16	4176.20	10.60	0.9431	0.0480	1.0000
P K	0.68	0.29	796.53	4.37	0.9608	0.7066	1.00
CoK	0.07	0.02	41.15	16.00	0.9798	0.9927	1.19

Fig. (9): EDX graph of Vitamin B_{12} like substance produced on Trypticase soy broth medium



Smart Quant Results

Element	Weight %	Atomic %	Net Int.	Error %	R	A	F
C K	67.25	73.29	5775.39	9.83	0.9369	0.1069	1.0000
N K	3.89	3.64	67.55	16.13	0.9410	0.0211	1.0000
O K	27.57	22.55	1227.77	10.88	0.9443	0.0431	1.0000
P K	1.20	0.51	616.34	4.22	0.9616	0.7252	1.0069
CoK	0.09	0.02	22.33	26.96	0.9803	0.9931	1.1945

Fig. (10): EDX graph of Vitamin B_{12} like substance produced on Mineral salt methanol Medium

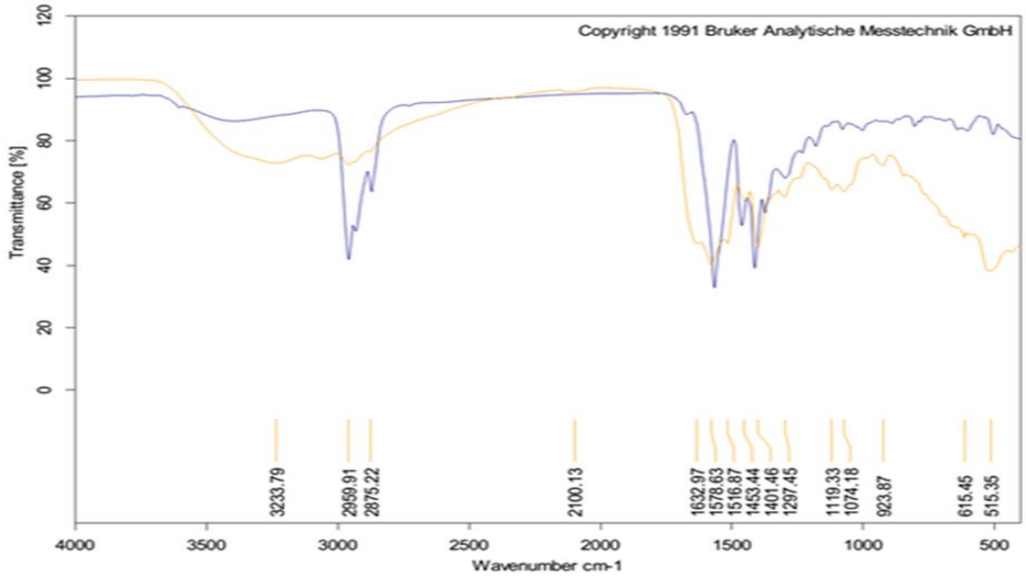


Fig. (11): IR spectrum of standard Vitamin B_{12} (Yellow color) and Vitamin B12 like substance from bacterial extract (blue color)

the present study, figure (1) exhibits the morphological shape of *Pseudomonas aeruginosa* M20. The results presented in Figures (2 - 8) show HPLC graphs. Figure (2) exhibits a peak with RT at 5.422 of standard vitamin B_{12}. Figure (3) presents the production of vitamin B_{12} on Trypticase soy broth medium and methanol-water as a solvent showing peak with RT at 5.522 which is closely related to standard Vitamin B_{12} and expected to be the most accepted medium and solvent for Vitamin B_{12} production. Figure (4) reveals the production of vitamin B_{12} on Trypticase soy broth medium and acetone as a solvent showing peak with RT at 4.793. Figures (5 and 6) show peaks with RT at 5.086 and 2.538 respectively while Figures (7 and 8) show the effect of gamma irradiation at doses 1.0 and 2.0 kGy on the bacterial extracts exhibiting peaks with RT.at 5.245 and 5.087 respectively.

The results of using the EDX technique shown in Figures (9 and 10) reveal 0.02 atomic percentages of cobalt in the purified extract. Figure (11) presents the IR spectrum of the standard vitamin B_{12} and the bacterial extract. This Figure obviously shows that both standard vitamin B_{12} and the bacterial extract have a similar behavior at wave numbers 1578.63 and 1453.44 cm^{-1}. Peaks that appeared at 3233 cm1, 2935 cm^1, 2875 cm^1, and 2100 cm1 are due to the stretching of the hydroxyl group (–OH), the C–H aromatic group, the C–H aliphatic group and C=O group. The result was in agreement with [21] **Which** reported that, the characterization of vitamin B_{12} was performed by using atomic force microscope (AFM), Fourier transform infrared-attenuated total reflectance (FTIR-ATR) vitamin B_{12} structure and MAGA monomer have N−H bands (amide II) at 3329 cm−1 , the aliphatic C−H stretching band at 2913 cm−1 and 2849 cm−1, carbonyl band 1761cm^{-1} −CH2 stretching band at 1435 cm^{-1}, asymmetric C−O−C stretching band at. Also [22] who reported that, FTIR spectrum of cobalamin is shown as a reference. The spectrum of VB_{12} shows two major parts. The broad peak at about 3400 cm1 is assigned to the O–H stretching of the hydroxyl groups attached to the surface. The second peak is in around of 1665 cm^1. The C=O stretching vibration due to propionamide side chain of VB_{12} caused the appearance of this intense peak at 1670 cm^1. Peak appeared at 3390 cm1, 3120 cm1, 2930 cm1, and 1665 cm1 are due to the stretching of the hydroxyl group (–OH), the C–H aromatic group, the C–H aliphatic group, and C=O group.

Table (3): Groups absorbing in IR region of bacterial extract

Range (cm^{-1})	Assignment
3996	O-H (free)
2999	O-H (very broad)
1749	C=O (saturated aldehyde)
1608	NH_2 scissoring (1°-amines)
1477	α-CH_2 bending
1430	α-CH_2 bending
1106	C-C-C bending
1019	C-C-C bending
895	=C-H & =CH_2

Table (4): Antimicrobial activity of Vitamin B_{12} extract against clinical bacterial and fungal strains

Inhibition zone n mm of 0.2 ml of irradiated and non-irradiated bacterial extract at doses level (0.0, 1.0 and 2.0 kGy)

Clinical isolates	0.0 kGy	1.0 kGy	2.0 kGy
Klebsiellae oxytoca	18	12	-ve
Citrobacter diversus	18	11	-ve
Enterobacter agglomerans	-ve	-ve	-ve
Escherichia coli	-ve	15	-ve
Staphylococcus aereus	-ve	19	-ve
Citrobacter freundii	-ve	-ve	-ve
Penicillium citrinum	15	-ve	-ve
Penicillium chrysogenum	18	30	-ve

The isolated strain was used for production of vitamin B_{12} -like substance; this was in agreement with the findings of [23] who reported that the new isolate, which designated as SP2, was identified to be a *Pseudomonas* strain based on the sequence homology of its 16S rDNA. The *Pseudomonas* strain SP2 had essential genes which responsible for vitamin B_{12} production such as *cobB* and *cobQ* and produced a similar amount of vitamin B_{12} -like substance (10.6 ± 0.05 μg/mL).

[24] reported that quantifying vitamin B_{12} by HPLC-MS exhibits that, the molecule only in cells that were grown aerobically and in the stationary phase. However, at exponential growth and in anaerobic conditions, vitamin B_{12} was detected below the technique detection limit.

In the present study γ-irradiation was used as stress factor to study their effect on the production of vitamin B_{12} from *Pseudomonas aeruginosa* M20. The purified extract of Vitamin B_{12} after Gamma irradiation exhibited a positive result at doses level of 1.0 and 2.0 kGy.

The results given in Table (3) exhibit the antimicrobial activities of vitamin B_{12} -like substance extracted from *Pseudomonas aeruginosa* M20 against Gram negative , Gram positive and fungal strains, the extract exhibited antimicrobial activity against *Klebsiellae oxytoca*, *Citrobacter diversus*, *Penicillium citrinum* and *Penicillium chrysogenum* showing inhibition zone (18,18,15 and 18 mm) respectively , on other hand don't exhibit antibacterail activity against *Enterobacter agglomerans*, *Escherichia coli* and *Citrobacter freundii* whilst exhibited positive results at dose of 1.0 kGy against *Klebsiellae oxytoca*, *Citrobacter diversus*, *Escherichia coli*, *Staphylococcus aereus* and *Penicillium chrysogenum*, showing inhibition zone (12,11,15,19 and 30 mm) respectively. On the other hand the extract did not exhibit antimicrobial activity at a dose of 2.0kGy

[26] Demonstrated that vitamins K and E had good synergistic activity with piperacillin/tazobactam, imipenem and doripenem against A. baumannii, whilst vitamins B1, B2 and B_{12} showed remarkable synergistic activity with linezolid against MRSA . **(27)** Found that**,** the strain S1 showed highest activity against the entire test organism, the inhibition zone was measured as (18, 20, 20 and 24 mm) against *E.coli, Klebsiella pneumoniae, Enterobactor faecalis* and *Staphylococcus aureus*. The strain S5 showed maximum activity (23, 22 mm) against *Escherichia coli* and *Enterobactor faecalis*.

CONCLUSION

The isolated and characterized *Pseudomonas aeruginosa* M20 produces a wide spectrum of bioactive compounds with applications in pharmaceutical like vitamin B_{12} which investigated at bench scale and further studies were recommended.

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ETHICAL APPROVAL

Not required.

FUNDING

None**.**

DATA AVAILABILITY

Not applicable**.**

CONSENT TO PARTICIPATE

Not applicable.

CONSENT TO PUBLISH

Not applicable.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Mahmoud Abdel Wahab Mahmoud: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Writing–review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization and Zahira Sayeid Tawfik Writing – review & editing, Writing – original draft.

REFERENCES

[1] Rehab Mohammed Elbargisy . Optimization of nutritional and environmental conditions for pyocyanin production by urine isolates of *Pseudomonas aeruginosa.* Saudi J Biol Sci. ; 28(1): 993–1000 (2021).

[2] Arat S., Bullerjahn G. S., Laubenbacher R.. A network biology approach to denitrification in Pseudomonas aeruginosa. PLoS ONE 10:e0118235. 10.1371/journal.pone.0118235 (2015).

[3] Torrents E. Ribonucleotide reductases: essential enzymes for bacterial life. Front. Cell. Infect. Microbiol. 4:52. 10.3389/fcimb.2014.00052 (2014).

[4] Warren M. J., Raux E., Schubert H. L., Escalante-Semerena J. C. The biosynthesis of adenosylcobalamin (vitamin B_{12}). *Nat. Prod. Rep.* 19, 390–412.

[5] Crespo A., Gavalda J., Julian E., Torrents E.. A single point mutation in class III ribonucleotide reductase promoter renders Pseudomonas aeruginosa PAO1 inefficient for anaerobic growth and infection. Sci. Rep. 7:13350. 10.1038/s41598-017-14051-2 (2017).

[6] Amada, Kazuhiro."Chapter 9. Cobalt: Its Role in Health and Disease". In Astrid Sigel, Helmut Sigel and Roland K. O. Sigel. Interrelations between Essential Metal Ions and Human Diseases. Metal Ions in Life Sciences. 13. Springer. pp. 295–320 (2013).

[7] Yazaki Y, Chow G, Mattie M. A single-center, double-blinded, randomized controlled study to evaluate the relative efficacy of sublingual and oral vitamin B-complex administration in reducing total serum homocysteine levels. J Altern Complement Med;12:881-5(2006).

[8] Kwak, C. S.; Lee, M. S.; Lee, H. J.; Whang, J. Y.; Park, S. C. "Dietary source of vitamin B_{12}intake and vitamin B_{12} status in female elderly Koreans aged 85 and older living in rural area". Nutrition Research and Practice. 4 (3): 229–234(2010).

[9] Riaz, Muhammad; Iqbal, Fouzia; Akram, Muhammad. "Microbial production of vitamin B_{12} by methanol utilizing strain of Pseudomonas specie". Pak J. Biochem. Mol. Biol. 1. 40: 5–10(2007).

[10] Høiby, N; Ciofu, O,; Bjarnsholt, T.. "Pseudomonas aeruginosa biofilms in cystic fibrosis". Future Microbiology. PubMed. 5 (11): 1663–74(2010).

[11] Schobert, Max; Jahn, Dieter ."Anaerobic physiology of Pseudomonas aeruginosa in the cystic fibrosis lung". International Journal of Medical Microbiology. 300 (8): 549–556(2010).

[12] Mavrodi D.V., Bonsall R.F., Delaney S.M., Soule M.J., Phillips G., Thomashow L.S. Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from Pseudomonas aeruginosa PAO1. J. Bacteriol.;183:6454–6465 (2001).

[13] Williams, P.; and Tomas, J.M. The pathogenicity of Klebsiella pneumoniae. Rev.Med.Microbiol.; 1;196–204(1990).

[14] Tawfik Zahira S. , El Shikh Hussein H. , Haroun Bakry M. , Yassin Mohamed M. , El Sonbty Sawsan M., Aman Gaber Zaki , and Mahmoud Abd Alwahab M. Biological and Histological Studies of Purified Product from Streptomyces janthinus M7 Metabolite The Open Access Journal of Science and Technology Vol. 3 , P.14 (2015).

[15] Isenberg, H.D. Clinical microbiology procedures handbook. Ameri. Soci. for Microbiol.;.p. 3.3.2-3.3.2.13(2004).

[16] Gephardt, P.; Murray R.G. E.; Costilow R. N.; Nester E.W, Wood W.A.; Krieg N.R. and Phillips G. B. Manual of Methods for General Bacteriology, ASM Press, Washington. V(2);185-189(1981).

[17] Augustine S K, Bhavsar S P, Baserisalehi M and Kapadnis B P. Isolation, Characterization and optimization of antifungal activity of an actinomycetev of soil orgigin;Indian J.Exp.Biol. 42 928 - 932(2004).

[18] Mohamed, H.F. The Biological Effects of Gamma Irradiation and / or Plant Extract (Neem) on the Greater Wax Moth, Galleria Mollenella. Eleventh Arab Conference on the Peaceful Uses of Atomic Energy, Khartoum, Sudan, 16-20(2012).

[19] Rather IA, Kim BC, Bajpai VK, Park YH . Self-medication and antibiotic resistance: Crisis, current challenges, and prevention. Saudi J Biol Sci. May;24(4):808-812(2017).

[20] Tumbarello M., Raffaelli F., Peghin M., Losito A.R., Chirico L., Giuliano G., Spanu T., Sartor A. Characterisation and risk-factor profiling of P.aeruginosa urinary tract infections: pinpointing those likely to be caused by multidrug-resistant strains. Int. J. Antimicrobial Agents. : 1059009 (2020).

[21] Seyed Mohsen ,Sadeghzadeh, a Rahele Zhianiab and Shokufe. KCC-1/GMSI/VB_{12} as a new nano catalyst for the carbonylative Suzuki–Miyaura cross coupling reaction. RSC Adv., 7, 32139,(2017).

[22] Duygu ÇIMEN and Adil DENIZLI ,.Development of Rapid, Sensitive, and Effective Plasmonic Nanosensor for the Detection of Vitamins in Infact Formula and Milk Samples. Photonic Sensors, DOI: 10.1007/s13320-020-0578-1 (2020).

[23] Frank Van der Aa, Steven Joniau, Marcel Van Den Branden, and Hein Van Poppe,. Metabolic Changes after Urinary Diversion, Adv Urol. (2011).

[24] Mariadhas V. A., Ritam S., Balaji S.S., Vinnod K.,Jin-dal –rae C.,Hyohak S.,Doyoung S. and Sunghoon P.,. Isolation of a novel *Pseudomonas* species SP2 producing vitamin B_{12} under aerobic condition. , Volume 18, Issue 1, pp 43–51(2013).

[25] Anna Crespo, Núria Blanco-Cabra, and Eduard Torrents, Aerobic Vitamin B_{12} Biosynthesis Is Essential for Pseudomonas aeruginosa Class II Ribonucleotide Reductase Activity During Planktonic and Biofilm Growth. Front Microbiol. ; 9: 986 (2018).

[26] Shakeel Shahzad , Muhammad Adnan Ashraf , Muhammad Sajid , Aqeel Shahzad , Azhar Rafique , Muhammad Shahid Mahmood .Evaluation of synergistic antimicrobial effect of vitamins (A, B1, B2, B6, B_{12}, C, D, E and K) with antibiotics against resistant bacterial strains. J Glob Antimicrob Resist. Jun;13:231-2369(2018) doi: 10.1016/j.jgar.2018.01.005.

[27] P.Selvakumar, G.Balamurugan, S.Viveka. Microbial Production of Vitamin B_{12} andAntimicrobial Activity of Glucose Utilizing MarineDerived Streptomyces Species. International Journal of ChemTech Research CODEN(USA): Vol.4, No.3, pp 976-982, (2012) IJCRGG ISSN : 0974-4290.