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STUDIES ON VITAMIN B_{12} PRODUCTION BY SOME SPECIES OF *Streptomyces* ISOLATED FROM SHARKIA GOVERNORATE SOILS

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ABSTRACT

One of the most alluring and fascinating molecules in the world of sciences, especially in foods and medicine is vitamin B_{12} (cyanocobalamin), which was originally discovered as the anti-pernicious anemia factor. In the current study, sixteen soil derived *Streptomyces* species isolated from Sharkia Governorate soils were examined for vitamin B_{12} production, only three of *Streptomyces* species produced vitamin B_{12} with good amounts were recorded after growing on a medium contains cobalt chloride (0.008 g per liter). The data showed that the three identified *Streptomyces* isolates as *S. griseus* (AK9), *S. nigrifaciens* (B9) and finally *S. aurofaciens* (Z3) have the ability for production of Vitamin B_{12} with 1.33, 1.06 and 2.66 µg/ml culture broth, respectively. The parameters controlling the biosynthetic process of vitamin B_{12} formation including using different raw materials in vitamin B_{12} fermentation media, pH values, incubation periods, temperatures and shaking rates (aeration) were investigated. Vitamin B_{12} in the collected fractions were measured by High Performance Liquid Chromatography (HPLC) method and also in order to confirm the existence of cyanocobalamin compounds in the samples, the best recoveries of vitamin B_{12} from hypersil gold C18 (10 µm, 100 x 4.66 mm) column were obtained when the column was eluted by methanol-water (22:78 V/V) as a mobile phase.

Key words: *Streptomyces* , Vitamin B_{12}, HPLC, raw materials, incubation periods, initial pH.

INTRODUCTION

Vitamins are regarded as organic compounds required in the diet in small amounts to perform specific biological functions for normal maintenance, optimum growth and health of the organisms. Vitamin B_{12} (cobalamin, anti-pernicious anemia factor) is a water soluble vitamin with a key role in the normal functioning of the brain and nervous system and for the formation of blood (Smith and Coman, 2014).

It is normally involved in the metabolism of every cell of the human body, especially fatty acid synthesis and energy production. This vitamin is provided as a supplement in many processed foods and is also available in vitamin pill form, including multivitamins. Vitamin B_{12} can be supplemented in healthy subjects also by liquid, transdermal patch, nasal spray, or injection and is available singly or in combination with other supplements (Shane, 2000; Loeffler, 2006). The structure of vitamin B_{12} (Cyanocobalamin) is $C_{63}\ H_{88}\ O_{14}\ N_{14}$ COP with molecular weight of 1355.42 dalton. Vitamin B_{12}, and specifically, its most common form cyanocobalamin, is odorless, tasteless and exists as a red powder or as red needle-like crystals. It is very hygroscopic in its anhydrous state but moisture can be removed at 105°C under reduced pressure. Also, this vitamin is soluble in water up to 1.25%, relatively soluble in lower alcohols and phenols, but insoluble in most other organic solvents. The crystals begin to darken at 210 -220°C and melt above 300°C, although cyanocobalamin in which CN-group is coordinate to the cobalt atom is the most common form of vitamin B_{12}, other forms also exists in which – OH (hydroxycobalamin), $-NO_2$ (nitrocobalamin), and $-HSO_3$ (sulfitcobalamin) are substituted for -CN group (Furlan, 1999;Khan and Easwaran, 2003; Carmen *et al.*, 2007).

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Also, this vitamin is the largest and most structurally complicated vitamin and can be produced industrially only through bacterial fermentation synthesis (Molina *et al.,* 2008) and being synthesized only by microorganisms and not by animals and plants.

The chemical synthesis of vitamin B_{12} on industrial scale is in principle very complicated and expensive. Therefore, it has been produced intracellularly or extracellularly on an industrial scale using the batch or fed batch process of microbial fermentation. Several microorganisms including those of the genera *Bacillus*, *Methanobacterium*, *Propionobactertium*, *Streptomyces* and *Pseudomonas* have been used to produce vitamin B_{12} on an industrial scale (Madigan *et al*., 2012). However, vitamin B_{12} has been isolated from fermentation broths of numerous microorganisms, including actinomycetes (Martens *et al.,* 2002). *Streptomyces* is the largest genus of Actinobacteria, over 500 species of *Streptomyces* bacteria have been described. *Streptomyces* is the most well known genus of Actinomycete family which always has been notified because of its ability to produce and secrete a large variety of industrial, medical, biotechnological and agricultural secondary metabolites (Fiedler *et al.,* 2005 and Madigan *et al.*, 2012).

Many reports pointed that many *Streptomyces* species have been reported as a good producers of the vitamin B_{12} with good amounts (Abd El-Meguid, 2000; Abu Elwafa, 2005; Atta *et al.,* 2008; Tehrani *et al*., 2009; Selvakumar *et al*., 2012).

The present study aimed to study vitamin B_{12} production by some *Streptomyces* species isolated from Sharkia Governorate soils and to study the parameters controlling vitamin B_{12} formation including raw materials, pH values, incubation periods, temperatures and shaking rates to show the optimum conditions for obtaining the highest yield of vitamin B_{12}.

MATERIALS AND METHODS

This study was carried out in the laboratory of Agricultural Microbiology Department, Faculty of Agriculture, Zagazig University, Egypt. The evaluation of efficient *Streptomyces* strains in producing vitamin B_{12} was carried out in the Central Laboratory, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Preparation of Standard Inoculums

The spores of the selected *Streptomyces* species were suspended with 10 ml of sterile distilled water and centrifuged for 10 min at 3000 rpm (Hopwood and Ferguson, 1985). One ml of the suspension containing about 16 x 10^9 cfu ml^{-1} was inoculated in 50 ml of starch nitrate broth medium in 250 ml flask. The inoculated flasks were incubated at 28 °C on a rotary shaker at 180 rpm for 4 days and the vegetative growth from each flask was used as standard inoculums.

Screening of *Streptomyces* Isolates Producing Vitamin B_{12}

Spores obtained from the slant culture of each isolate were inoculated into 50 ml of the activation medium in 250 Erlenmeyer flask and incubated on a rotary shaker (180 rpm) at 24-26°C for 2 days. Five ml of previous vegetative growth medium were transferred into 50 ml screening medium (modified starch nitrate fermentation medium) in 250 Erlenmeyer flask and incubated under the same conditions for 7 days (Saunders *et al*., 1952). After incubation period, vitamin B_{12} was extracted according to the method described by Hafez (1993) as follows: The growth obtained from each strain was centrifuged for 10 min at 3500 rpm, the pellet was washed with distilled sterile water for many times and resuspended with 10 ml of 0.2M acetate buffer (pH 5) containing 0.001% potassium cyanide, the suspension was autoclaved for 20 min at 121°C and filtered by syringe filters Mixed Cellulose Esters (MCE) membrane, pore size 0.45 µm which were purchased from Corrigtwohill Co., Cork, Ireland. The vitamin B_{12} probability produced was identified and assayed (µg ml^{-1}) using High Performance Liquid Chromatography (HPLC) apparatus equipped with a surveyor quaternary pump with intel. vacuum degasser and equipped with Hypersil gold C18 (10 µm, 100 x 4.6 mm) column eluted by methanol-water (22:78 V/V) as a mobile phase (Rada *et al.,* 2004) and detection with a photodiode array detector at 210 nm (PAD Thermo Scientific Co., USA).

The standard solution of vitamin B_{12} was prepared by 10 mg of cyancobalamin reference standard, dissolved with 50 ml mobile phase in

100 ml volumetric flask, the volume was completed with mobile phase to50, 25, 12.5, 6.25, 3.125, 1.56 µg/ml. Twenty µL of each previous standard solution and filtered suspension from each *Streptomyces* isolates were injected separately in HPLC system and data were recorded.

Effect of Different Concentrations of Raw Materials

Molasses and oatmeal extracts at the different concentrations of 5, 10, 15 and 20 ml/L and cheese whey at various rates of 20, 30 and 40 ml / L were added separately instead of starch in modified starch nitrate fermentation medium and initial pH was adjusted to 7 by phosphate buffer (British pharmacopoeia, 2000). 50 ml of every treatment in 250 ml flask was sterilized. Then the inoculated flasks were incubated at 28°C on rotary shaker at 180 rpm for 7 days. The vegetative growth of the efficient selected *Streptomyces* species obtained from each flask was washed by sterile distilled water for many times, dried at 70°C for 24 hr., and then weighted. Vitamin B_{12} was extracted according to Hafez (1993) and determined by HPLC method recommended by Rada *et al.* (2004). The average of the amount of vitamin B_{12} production was determined and the results were expressed as a µg vitamin B_{12} / ml culture broth.

Growth Conditions affecting Vitamin B_{12} Production

The suitable concentration of each raw material used (molasses, oatmeal and cheesy whey) was added separately to the modified starch nitrate fermentation medium instead of starch. The effect of incubation periods (4, 5, 6 and 7 days); initial pH (5, 6, 7 and 8); incubation temperatures (24, 28, 32 and 36°C) and shaking rates (0, 160, 180, 200 rpm) on growth and vitamin B_{12} production were studied. After each of aforementioned experiment, the suitable factor giving highest yield of vitamin B_{12} was applied as a control in the subsequent experiment.

Vitamin B_{12} was extracted from the vegetative growth by the method as recommended by Hafez (1993) and was determined by using HPLC method recommended by Rada *et al.* (2004).

RESULTS AND DISCUSSION

Production of Vitamin B_{12} by The most Efficient *Streptomyces* species

The *Streptomyces* is one of the few microorganisms producing significant amount of vitamin B_{12} (Tehrani *et al.*, 2009). Sixteen selected isolates of *Streptomyces* were examined for the production of vitamin B_{12}. Only three isolates of *Streptomyces* species from the sixteen isolates produced vitamin B_{12} with good amounts after growing on a medium contains cobalt chloride with amount of 0.008 g per liter.

The data in Table 1 and Figs. 1 and 2 showed that three identified *Streptomyces* isolates as *S. aurofaciens* (Z3), *S. nigrifaciens* (B9) and finally *S. griseus* (AK9) have the ability for the production of vitamin B_{12} with 2.66, 1.06 and 1.33, 1.06 µg/ml, respectively and 133, 53.0 and 66.5 µg per 50 ml culture broth.

The data in Table 1 and Figs. 1 and 2 showed also that *Streptomyces aureofaciens* (Z3) has the highest ability for production of vitamin B_{12} with total yield of 2.66 µg/ml after 7 days at pH 7, 28 ±2°C at 180 rpm while *Streptomyces nigrifaciens* (B9) had the lowest production of vitamin B_{12} with total yield of 1.06 µg/ml culture broth. On the other hand, the other thirteen isolates had almost no ability to produce vitamin B_{12}.

In addition, the results cleared that 19% of *Streptomyces* species from 16 species produced vitamin B_{12} in the range of 1.06 to 2.66 µg/ml culture broth. Almost similar results were reported by Salama and Kamal (1983). They found that 21% of isolates produced vitamin B_{12} in the range of 1.0 to 4.0 µg/ml. But, Ibrahim (1989) found that 45% of *Streptomyces* isolates among 91 isolates produced vitamin B_{12} in the range of 0.7 to 1.1 µg/ml. While, Abd El-Meguid (2000) found that *Streptomyces griseus* produced vitamin B_{12} in a good amount (18.0 µg/ml). Also, Selvakumar *et al.* (2012) found that among fifteen *Streptomyces* isolates, only seven produced vitamin B_{12} (46.6 %) and quantified its concentration range from 1.9 to 45.3µg per ml.

On the other hand, Atta *et al.* (2008) reported that only one actinomycete isolate (well identified as *Streptomyces halstedii*, AZ-8A) out of 33 actinomycetes isolates produced significantly higher yield of vitamin B_{12} (37.7 µg/ ml).

Table 1. Production of vitamin B_{12} by the most efficient species of *Streptomyces* in modified starch nitrate fermentation medium

Parameters	*Sterptomyces* species															
	Z3	Z10	Z14	Z15	B2	B9	R1	H1	H7	AK4	AK9	S11	S12	AH1	AH6	AH12
Vitamin B_{12} yield (µg/ ml culture broth)	2.66	0.00	0.00	0.00	0.00	1.06	0.00	0.00	0.00	0.00	1.33	0.00	0.00	0.00	0.00	0.00
Total vitamin B_{12} (µg / 50 ml culture broth)	133.0	0.00	0.00	0.00	0.00	53.0	0.00	0.00	0.00	0.00	66.5	0.00	0.00	0.00	0.00	0.00

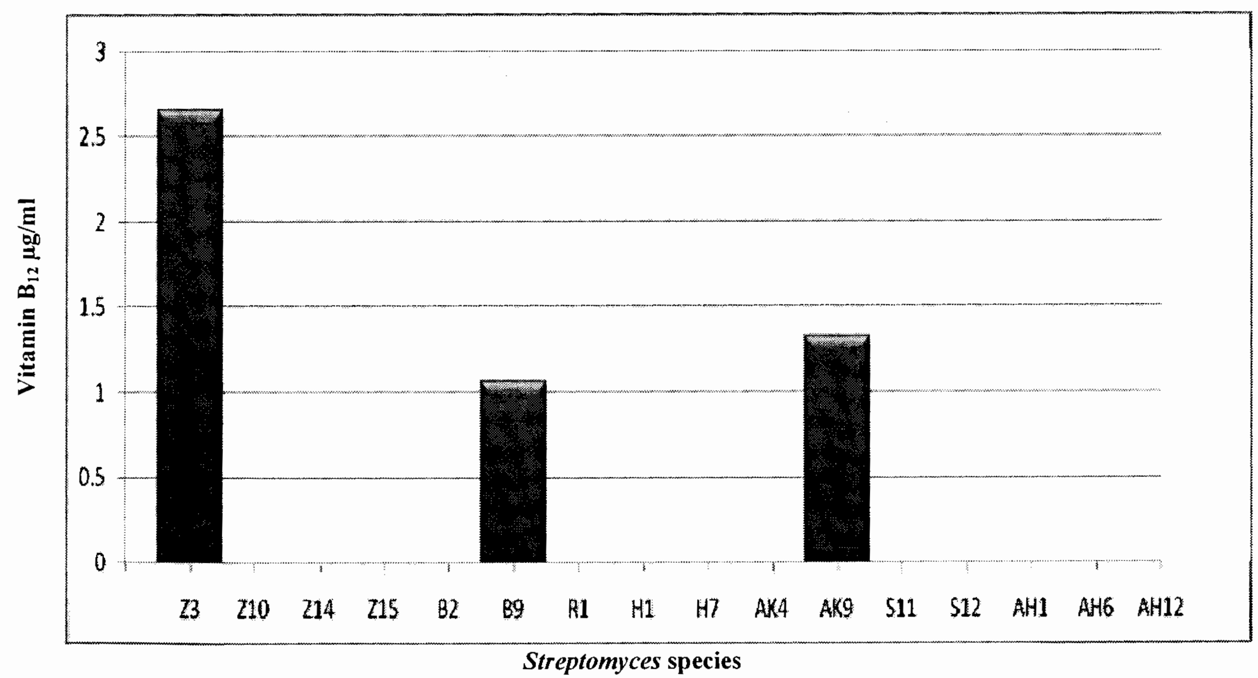

Fig. 1. The production of vitamin B_{12} by the most efficient *Streptomyces* species

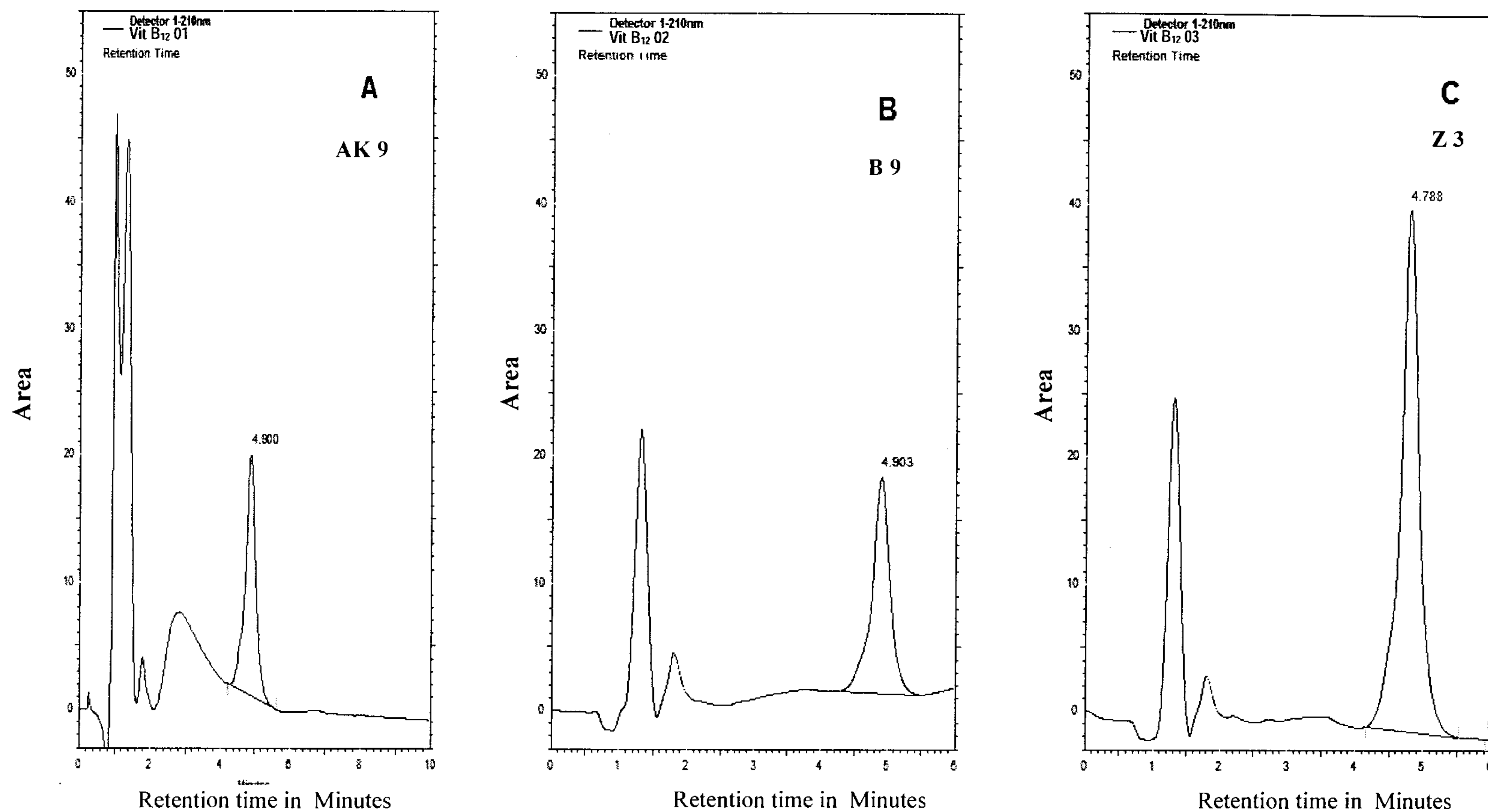

Fig. 2. Vitamin B_{12} peaks detection by HPLC detector at 210 nm showing the retention time of each peak for the most efficient *Streptomyces* species producing vitamin B_{12}; A) *S. griseus* (Ak9), B) *S. nigrifaciens* (B9) and C) *S. aureofaciens* (Z3)

Factors Affecting the Production of Vitamin B_{12}

Raw materials

Different concentrations of some raw materials, *i.e.*, molasses (5, 10, 15 and 20 g/L); oatmeal (5, 10 and 15 g /L) and cheese whey (20,'30 and 40 ml/L) were separately added in modified fermentation medium. Data in Table 2 and Fig. 3 a,b,c clearly show that the optimum concentrations for vitamin B_{12} production using molasses, oatmeal and cheesy whey were 15, 15 g / L and 40 ml / L , respectively.

With regard to molasses, the results in Table 2 and Fig. 3a showed that the highest yield of vitamin B_{12} produced by *S. griseus* (AK9), *S. nigrifaciens* (B9) and *S. aureofaciens* (Z3) were obtained in modified fermentation medium containing 15 g/L molasses, being 283.5, 206.5 and 518.5 µg per 50 ml culture broth, respectively. The increasing percentage of vitamin B_{12} produced at 15 g/L molasses were 426.3%, 389.9% and 389.8%, respectively as compared with the control treatments. Thus the concentration of 15 g/L molasses was selected for subsequent study.

As for oatmeal extract, results recorded in Table 2 and Fig. 3b revealed also that the highest yields of vitamin B_{12} produced by *S. griseus* (AK9), *S. nigrifaciens* (B9) and *S. aureofaciens* (Z3) were 145.5, 108.5 and 290 µg per 50 ml culture broth, respectively in modified fermentation medium containing 15 g/ L oatmeal extract, followed in decreasing order by 133.5, 110.0 and 306.5 ; 83.5, 71.5 and 163 µg per 50 ml culture broth, respectively in the same medium containing 10 and 5 g / L oatmeal extract, respectively.

Concerning cheese whey, it is clear from data in Table 2 and Fig. 3c that the highest yields of vitamin B_{12} produced by *S. griseus* (AK9), *S. nigrifaciens* (B9) and *S. aureofaciens* (Z3) were 79.0, 63.5 and 167.0 µg per 50 ml culture, respectively in modified fermentation medium containing 40 ml / L cheese whey, followed in decreasing order by 53.5, 43.5 and 80.5; 43.0, 30.0 and 62.5 µg per 50 ml culture, respectively in the same medium containing 30 and 20 ml/L cheese whey, respectively. The reduction in vitamin B_{12} yield at the lowest concentration at 20 and 30 ml of cheese whey per liter as compared to control treatment might be attributed to the chemical composition of this waste byproduct (rich in lactose sugar and very poor in glucose sugar) as well as this vitamin is known as a fermentative production compound by microorganisms mainly from glucose.

The present results concluded that the optimum concentration of different raw materials added to the fermentation medium was found to be 15 g/L molasses for *S. griseus* (AK9), *S. nigrifaciens* (B9) and *S. aureofaciens* (Z3).

The present results are in agreement with the results reported by Abou-Zied and Yousef (1971) and Abu Elwafa (2005). They found that microbial production of vitamin B_{12} in highly most amount was found in various media containing molasses. However, early studies by Shteinbery *et al.* (1985) stated that molasses has contained betaine and this compound stimulated the vitamin B_{12} production by microorganisms.

Effect of Incubation Period

Data presented in Table 3 and Fig. 4 showed that the production of vitamin B_{12} gradually increased in modified fermentation medium containing 15 g/L molasses with the increasing of incubation period until the 5^{th} to 6^{th} day, then slightly decreased up to the 7^{th} day. The highest yield of vitamin B_{12} was 299 µg per 50 ml culture media after 6 days for *S. griseus* (AK 9) then slightly decreased to 283.5 µg per 50 ml culture broth on the 7^{th} day, while the lowest yields of vitamin B_{12} were 275 and 174 .5 µg per 50 ml culture media after 5 and 4 days, respectively.

Data also recorded in Table 3 and Fig. 4 showed that the highest yield of vitamin B_{12} was 293 µg per 50 ml culture broth after 5 days for *S. nigrifaciens* (B9), then slightly decreased to 253 and 206.5 µg per 50 ml culture broth on the 6^{th} and the 7^{th} day, respectively while the lowest yield of vitamin B_{12} was 155.5 per 50 ml culture on the 4^{th} day.

Table 2. Production of vitamin B_{12} by efficient *Streptomyces* species in modified fermentation medium containing different raw materials at various concentrations

Streptomyces species			*S. griseus* (AK9)		*S. nigrifaciens* (B9)		*S. aureofaciens* (Z3)	
Paramaters *			Vitamin B_{12} yield (µg/ ml culture)	Total vitam. B_{12} (µg/50 ml culture)	Vitamin B_{12} yield (µg/ml culture)	Total vitam. B_{12} (µg/50 ml culture)	Vitamin B_{12} yield (µg/ml culture)	Total vitam. B_{12} (µg/50 ml culture)
Control medium**			1.33	66.5	1.06	53.0	2.66	133.0
Raw materials	Molasses (ml/liter)	5	1.77	88.5	1.42	71.0	3.85	192.5
		10	3.19	159.5	2.58	129.0	6.89	344.5
		15	5.67	283.5	4.13	206.5	10.37	518.5
		20	4.12	206.0	3.03	151.5	8.77	438.5
	Oatmeal (g/liter)	5	1.67	83.5	1.43	71.5	3.26	163.0
		10	2.67	133.5	2.20	110.0	6.13	306.5
		15	2.91	145.5	2.17	108.5	5.80	290.0
	Cheesy whey (ml/liter)	20	0.86	43.0	0.60	30.0	1.25	62.5
		30	1.07	53.5	0.78	43.5	1.61	80.5
		40	1.58	79.0	1.27	63.5	3.34	167.0

* After the 7th day of incubation at 28 ± 2 °C, under submerged culture condition (180 rpm)

** Control medium : Starch nitrate broth

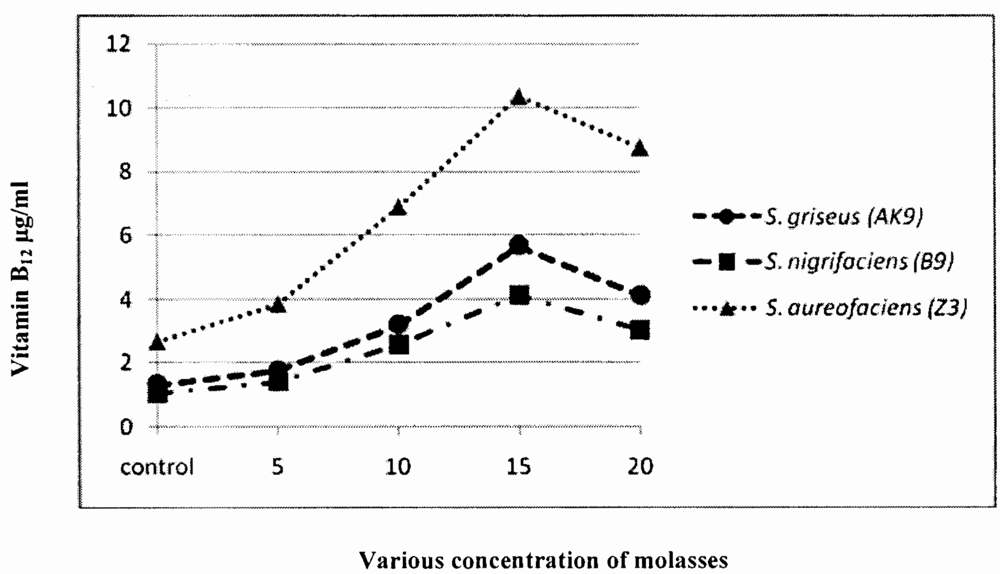

Fig. 3a. Production of vitamin B_{12} by the most efficient *Streptomyces* species in modified fermentation medium containing different concentrations of molasses

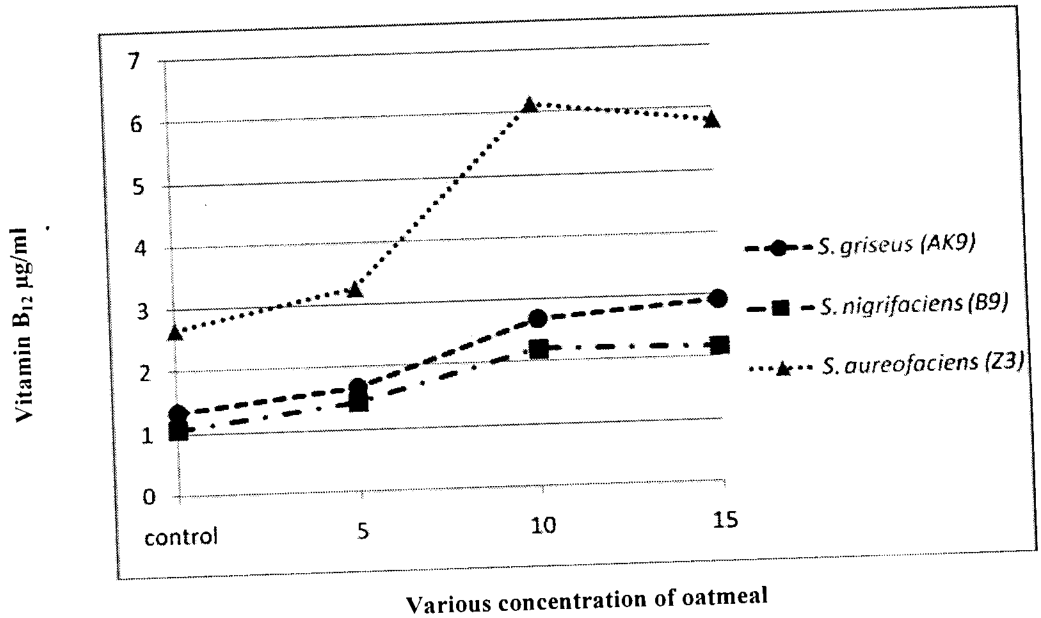

Fig. 3$_b$. Production of vitamin B_{12} by efficient *Streptomyces* species in modified fermentation medium containing different concentrations of oatmeal

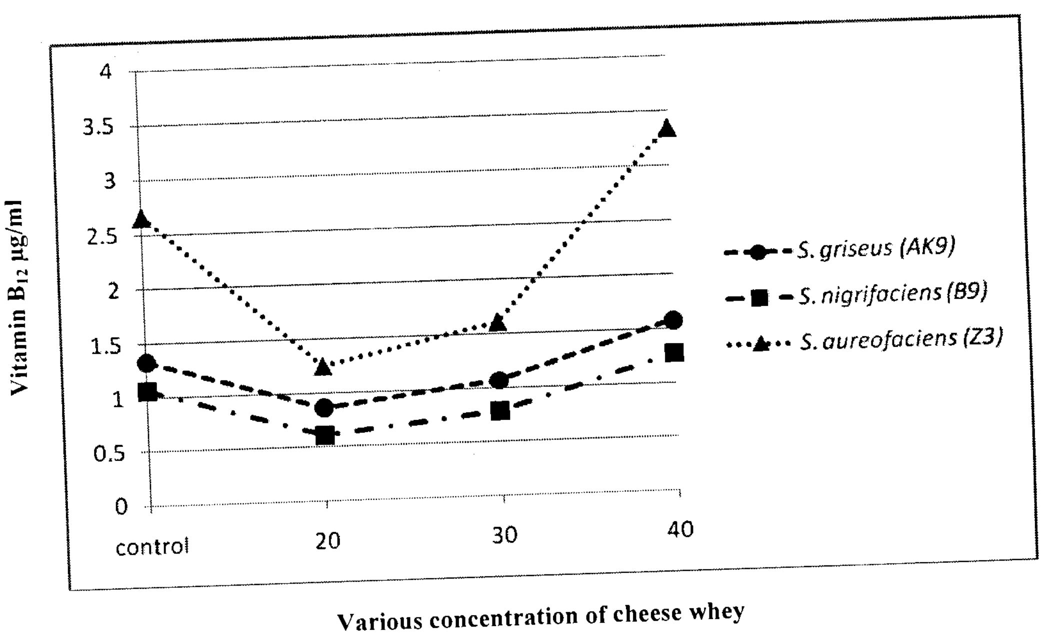

Fig. 3$_c$. Production of vitamin B_{12} by efficient *Streptomyces* species in modified fermentation medium containing different concentrations of cheese whey

Table 3. Effect of incubation periods on the production of Vitamin B_{12} by the most efficient *Streptomyces* species

Streptomyces species		*S. griseus* (AK9)		*S. nigrifaciens* (B9)		*S. aureofaciens* (Z3)	
Paramaters		Vitamin B_{12} yield (µg/ ml culture)	Total vitam. B_{12} (µg/50 ml culture)	Vitamin B_{12} yield (µg/ ml culture)	Total vitam. B_{12} (µg/50 ml culture)	Vitamin B_{12} yield (µg/ ml culture)	Total vitam. B_{12} (µg / 50 ml culture)
Incubation period (Days)	4	3.49	174.5	3.11	155.5	7.33	366.5
	5	5.51	275.5	5.86	293.0	9.12	456.0
	6	5.98	299.0	5.06	253.0	11.55	577.5
	7*	5.67	283.5	4.13	206.5	10.37	518.5

* Control treatment at pH 7, temperature 28 ± 2 °C, shaking rate 180 rpm and modified fermentation medium containing 15 g/ L molasses

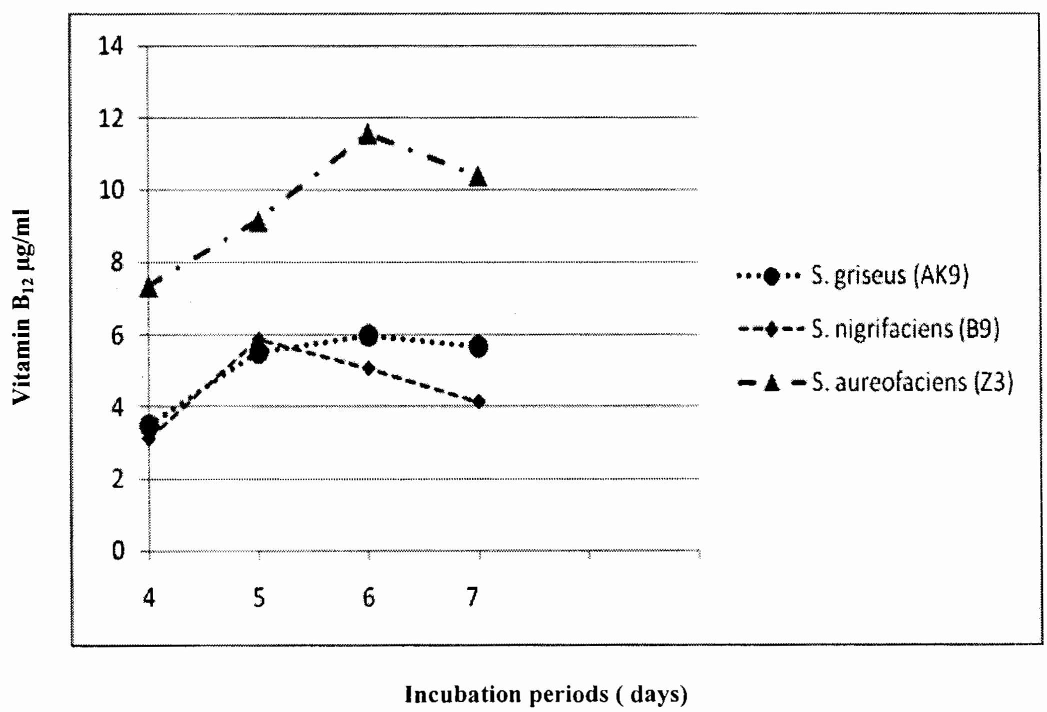

Fig. 4. Effect of incubation periods on the production of Vitamin B_{12} by the most efficient *Streptomyces* species

In addition, data recorded in Table 3 and Fig. 4 showed that the highest yield of vitamin B_{12} was 577.5 µg per 50 ml culture broth after 6 days for *S. aueofaciens* (Z3), then slightly decreased to 518.0 µg per 50 ml culture broth on the 7[th] day respectively, while the lowest yield of vitamin B_{12} was 366.5 per 50 ml culture on the 4[th] day.

In this respect, Abd El-Meguid (2000) found that the highest yield of vitamin B_{12} by *Streptomyces griseus* in fermentation medium was on the 5[th] day. On the other hand, Abu Elwafa (2005) determined that the highest yield of vitamin B_{12} obtained by *Streptomyces baarnesis* was on the 4[th] day. Also, Atta *et al.* (2008) reported that the maximum biosynthesis of vitamin B_{12} was recorded with an incubation period of 96 hours (4 days) for a mixed culture of *Streptomyces halstedii* and *Bacillus firmus*.

Effect of Initial pH

The effect of initial pH of the medium on the vitamin B_{12} yield produced by the selected *Streptomyces* species grown in modified fermentation medium containing 15 g/L molasses and after 5 days (*S. nigrifaciens*) and 6 days (*S. griseus* and *S. aureofaciens*) of incubation period are presented in Table 4 and Fig. 5.

It is obvious from data in Table 4 and Fig. 5 that the production of vitamin B_{12} gradually increased with increasing the initial pH degree of the modified fermentation medium until pH 7, then the vitamin B_{12} yield decreased thereafter.

The highest yields of vitamin B_{12} were 299.0, 293.0 and 577.7 µg per 50 ml of culture broth by *S. griseus* (AK9), *S. nigrifaciens* (B9) and *S. aureofaciens* (Z3), respectively at the level of pH 7, while the lowest yields of vitamin B_{12} were 167.5, 99.5 and 354.0 µg per 50 ml of culture media by *S. griseus* (AK9), *S. nigrifaciens* (B9) and *S. aureofaciens* (Z3), respectively at the level of pH 8. Thus, the present results concluded that the highest yield of vitamin B_{12} was at initial pH 7which was selected for subsequent study.

These results are in accordance with those obtained by Ibrahim (1989) who reported that the maximum production of vitamin B_{12} was around pH 7, but pH 4 and 9 resulted in a very marked effect. Also Abd El-Meguid (2000) found that the range of pH from 5 to 8 was suitable for producing a high yield of vitamin B_{12} by *Streptomyces griseus* in fermentation medium.

Abu Elwafa (2005) also reported that maximum production of vitamin B_{12} by *Streptomyces baarnesis* strain SW1 was around pH 7 in fermentation medium containing 15 g/L molasses.

On the other hand, Atta *et al.* (2008) reported that the maximum production of vitamin B_{12} by *Streptomyces halstedii* was around pH 5.5.

Effect of Incubation Temperature

Temperature is a limiting factor for the production of vitamin B_{12} by microorganisms. This factor was studied to select the optimum incubation temperature degree for the producing of the maximal yield of vitamin B_{12} by the experimental *Streptomyces* species.

The effect of incubation temperature of the medium on the vitamin B_{12} yield produced by the selected *Streptomyces* species grown in modified fermentation medium containing 15 g/L molasses and after 5 days (*S. nigrifaciens*) and 6 days (*S. griseus* and *S. aureofaciens*) of incubation period and at pH level 7 are presented in Table 5 and Fig. 6.

It is obvious from data in Table 5 and Fig. 6 that the production of vitamin B_{12} gradually increased with increasing the incubation temperature of the modified fermentation medium until 28 – 32°C, then the vitamin B_{12} yield decreased thereafter.

Data in Table 5 and Fig. 6 obviously showed that the optimum incubation temperature was at 28 - 32°C to produce the highest yields of vitamin B_{12}. The highest yield of vitamin B_{12} obtained by *S. griseus* (Ak9) and *S. aureofaciens* (Z3) were 299 and 577.5 µg per 50 ml culture broth, respectively at 28°C of incubation temperature, while the highest yield of vitamin B_{12} obtained by *S. nigrafaciens* (B9) was 294.5 µg per 50 ml culture broth at 32°C of incubation temperature.

Table 4. Effect of initial pH values on the production of vitamin B_{12} by the most efficient *Streptomyces* species

Streptomyces species		*S. griseus* (AK9)		*S. nigrifaciens* (B9)		*S. aureofaciens* (Z3)	
Total vitam. B_{12} (µg/50 ml culture)		Vitamin B_{12} yield (µg/ml culture)	Total vitam. B_{12} (µg/50 ml culture)	Vitamin B_{12} yield (µg/ml culture)	Total vitam. B_{12} (µg/50 ml culture)	Vitamin B_{12} yield (µg/ml culture)	Total vitam. B_{12} (µg/50 ml culture)
Initial pH valve	5	5.38	269.0	2.30	115.0	6.55	327.5
	6	5.39	269.5	5.59	279.5	9.03	451.5
	7*	5.98***	299.0	5.86**	293.0	11.55***	577.5
	8	3.35	167.5	1.99	99.5	7.08	354.0

* Control initial pH Value

** At 28 ±2 °C for 5 days, fermentation medium containing 15 g /L molasses and shaking rate 180 rpm

*** At 28 ±2 °C for 6 days, fermentation medium containing 15 g /L molasses and shaking rate 180rpm

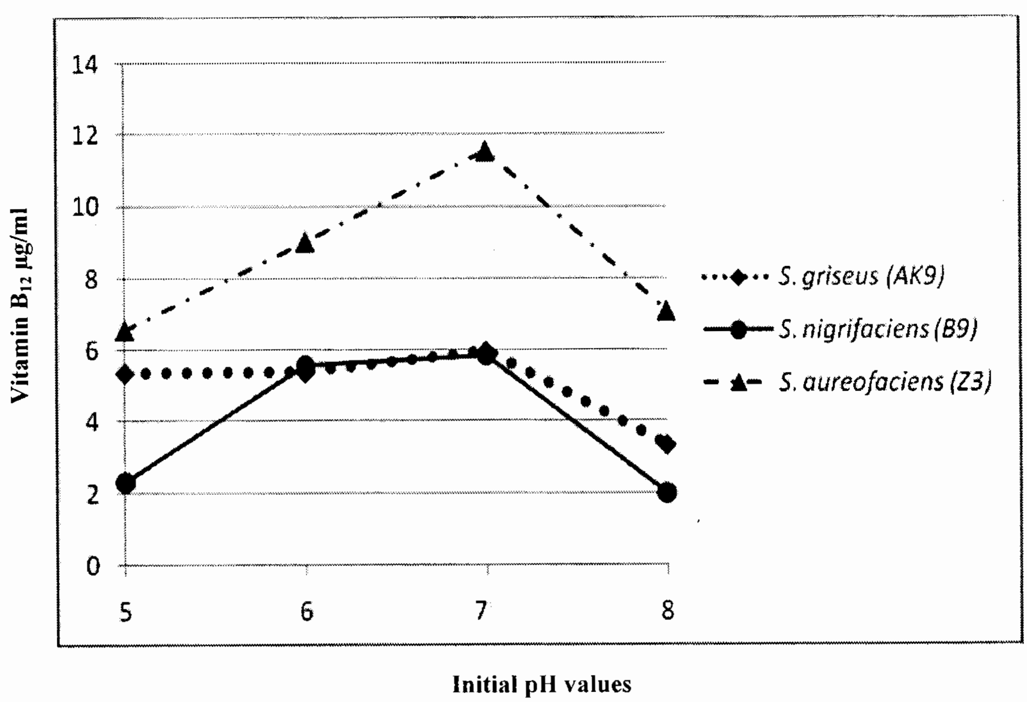

Fig. 5. Effect of initial pH on the production of vitamin B_{12} by the most efficient *Streptomyces* species

Table 5. Effect of incubation temperature on the production of vitamin B_{12} by the most efficient *Streptomyces* species

Streptomyces species		*S. griseus* (AK9)		*S. nigrifaciens* (B9)		*S. aureofaciens* (Z3)	
Total vitam. B_{12} (µg/ 50 ml culture)		Vitamin B_{12} yield (µg/ ml culture)	Total vitam. B_{12} (µg/50 ml culture)	Vitamin B_{12} yield (µg/ml culture)	Total vitam. B_{12} (µg/50 ml culture)	Vitamin B_{12} yield (µg/ml culture)	Total vitam. B_{12} (µg/50 ml culture)
Temperature degree °C	24	3.28	164.0	3.33	166.6	6.29	314.5
	28*	5.98***	299.0	5.86**	293.0	11.55***	577.5
	32	4.47	223.5	5.89	294.5	8.81	440.5
	36	2.38	119.0	4.91	245.5	7.59	379.5

* Control temperature degree

** At pH 7 for 5 days, fermentation medium containing 15 g/ L molasses and shaking rate 180 rpm

*** At pH 7 for 6 days, fermentation medium containing 15 g /L molasses and shaking rate 180 rpm

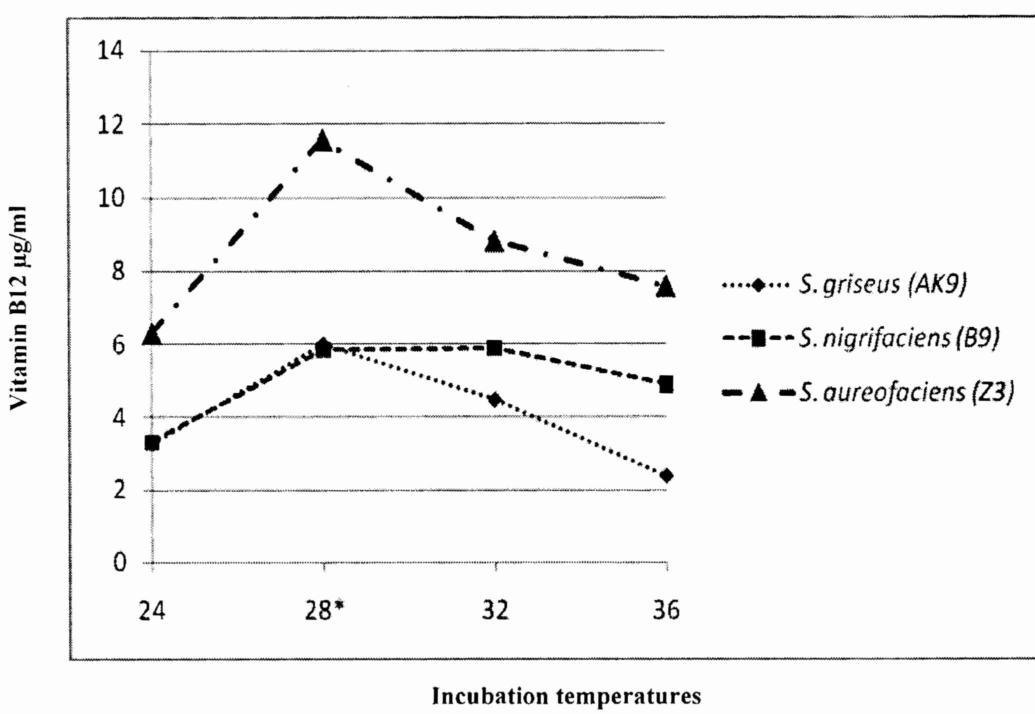

Fig. 6. Effect of temperature on the production of vitamin B_{12} by the most efficient *Streptomyces* species

These results are in consist with those obtained by Abd El-Meguid (2000) who mentioned that optimum temperature for B_{12} production was at 30°C by *Streptomyces griseus* and *Bacillus megaterium*, also Abu Elwafa (2005) reported that maximum production of vitamin B_{12} by *Streptomyces baarnesis* strain SW1 was around pH 6 in fermentation medium containing 15 g/L molasses after 6 days at incubation temperature of 28°C.

On the other hand, Atta *et al.* (2008) reported that the maximum biosynthesis of vitamin B_{12} was recorded within an incubation temperature of 35°C for a mixed culture of *Streptomyces halstedii* and *Bacillus firmus*.

Effect of Shaking Rate

The effect of shaking rate of the medium on the vitamin B_{12} yield produced by the selected *Streptomyces* species grown in modified fermentation medium containing 15 g/L molasses and after 5 days (*S. nigrifaciens*) and 6 days (*S. griseus* and *S. aureofaciens*)of incubation period and at pH level 7 and incubation temperature ranged between 28 – 32°C are presented in Table 6 and Fig. 7.

It is obvious from the data in Table 6 and Fig. 7 that the production of vitamin B_{12} gradually increased with increasing of shaking rates of the modified fermentation medium until 180 – 200 rpm. The highest yields of vitamin B_{12} obtained by *S. griseus* (Ak9) and *S. aureofaciens* (Z3) were 299 and 577.5 µg per 50 ml culture media, respectively at 180 rpm, while the highest yield of vitamin B_{12} obtained by *S. nigrafaciens* (B9) was 296.5 µg per 50 ml culture media at 200 rpm. While static incubation resulted in almost no production of the vitamin B_{12} for selected species.

These data are in agreement with the findings of Abd El- Meguid, (2000) who reported that the maximum productivity of vitamin B_{12} was at 200 rpm for *Streptomyces griseus*, while static incubation resulted in almost no production of the vitamin B_{12}. Also, Abu Elwafa (2005) studied the effect of aeration on the productivity of *Streptomyces baarensis* SW1 and he found that the maximum productivity of vitamin B_{12} was at 200 rpm, he also found that static incubation resulted in almost no production of vitamin B_{12}.

Table 6. Effect of shaking rate on the production of vitamin B_{12} by the most efficient *Streptomyces* species

Streptomyces species		*S. griseus* (AK9)		*S. nigrifaciens* (B9)		*S. aureofaciens* (Z3)	
Total vitam. B_{12} (µg / 50 ml culture)		Vitamin B_{12} yield (µg/ml culture)	Total vitam. B_{12} (µg /50 ml culture)	Vitamin B_{12} yield (µg/ml culture)	Total vitam. B_{12} (µg/50 ml culture)	Vitamin B_{12} yield (µg/ml culture)	Total vitam. B_{12} (µg/50 ml culture)
Shaking rate (rpm)	0	0.00	0.00	0.00	00.0	0.00	0.00
	160	4.37	218.5	3.52	176.0	8.64	432.0
	180*	5.98**	299.0	5.89***	293.0	11.55****	577.5
	200	5.52	276.0	5.93	296.5	10.70	535.0

* Control shaking rate

** At 28 °C, pH 7 for 5days and fermentation medium containing 15 g /L molasses

*** At 32 °C ,pH 7 for 6 days and fermentation medium containing 15 g /L molasses

**** At 28 °C, pH 7 for 6 days and fermentation medium containing 15 g/ L molasses

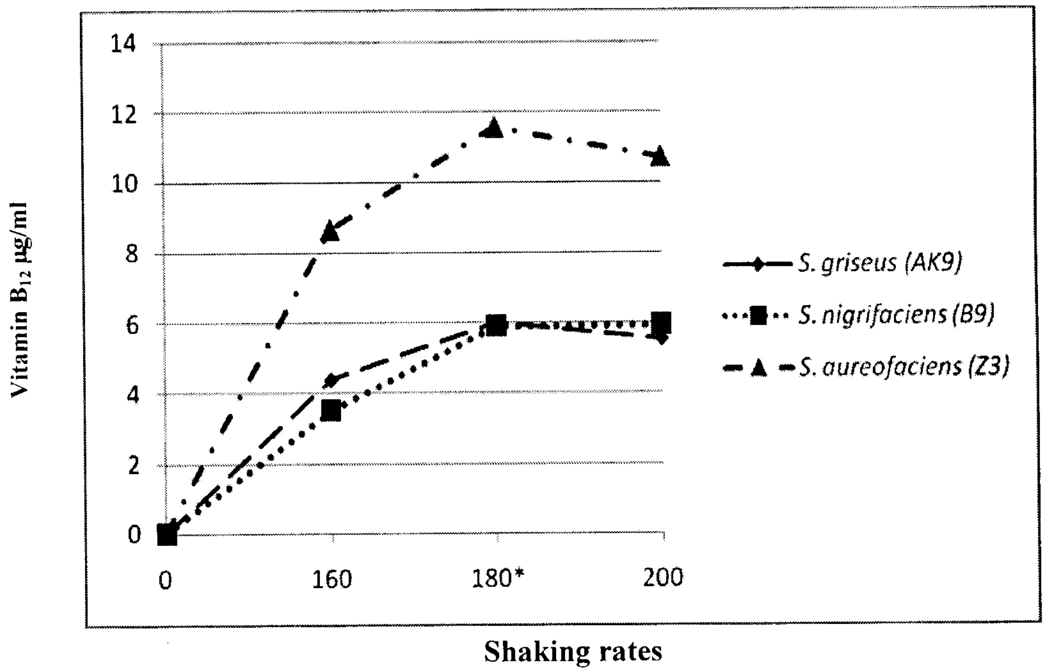

Fig. 7. Effect of shaking rate on the production of vitamin B_{12} by the most efficient *Streptomyces* species

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دراسات على إنتاج فيتامين ب ١٢ بواسطة بعض الأنواع التابعة لجنس الإستربتوميسيس المعزولة من أراضي محافظة الشرقية

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يعتبر فيتامين ب ١٢(السيانوكوبالأمين) واحد من أهم الجزيئات وأكثرها دهشة وروعة في عالم العلوم والغذاء والطب والذي تم اكتشافه بالأساس كأحد العوامل الهامة المضادة للأنيميا، تم دراسة ستة عشر عزلة تابعة لجنس الاستربتوميسيس معزولة من أراضي محافظة الشرقية لاختبار قدرتها علي إنتاج فيتامين ب ١٢، وجد فقط ثلاث أنواع لها القدرة علي إنتاج فيتامين ب١٢ بكميات جيدة في بيئة تحتوي علي ٠,٠٠٠٨ جرام من كلوريد الكوبالت، ولقد أوضحت النتائج أن هذه الأنواع الثلاثة المعرفة هي*S. griseus (*AK9*), S. nigrifaciens (*B9*) ,S. aurofaciens (*Z3*)* كانت لديهم القدرة علي إنتاج فيتامين ب١٢ بكميات ١,٣٣ و ١,٠٦ و ٢,٦٦ ميكروجرام لكل مل بيئة علي التوالي، كانت العوامل التي تؤثر علي الإنتاج الحيوي لفيتامين ب١٢ و التي تشمل استخدام مواد خام مختلفة، مدة التحضين، درجة تركيز أيون الأيدروجين، درجة حرارة التحضين وسرعة رج البيئة (التهوية) قد تمت دراستها بالتفصيل لمعرفة أفضل ظروف انتاج فيتامين ب١٢ بهذه الأنواع البكتيرية، تم قياس كمية فيتامين ب ١٢ المتحصل عليها عن طريق التحليل بواسطة جهاز الـ HPLC و أيضا لتأكيد وجود السيانوكوبالأمين في العينة فانه تم فصل وقياس فيتامين ب ١٢ في عامود من نوع Hypersil gold C18 مع استخدام الميثانول – الماء بنسبة (٢٢:٧٨ حجم/حجم) كعامل فصل متحرك.

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