Production of vitamin B_{12} via microbial strains isolated from marine and food sources in Egypt

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Background
Vitamin B_{12} is a very important water-soluble vitamin, which was first isolated from the liver as an anti-pernicious anemia factor. The sole source of vitamin B_{12} is the animal-based food. It has a complicated structure and requires expensive multi-steps to be synthesized chemically. Intriguingly, vitamin B_{12} can be produced through microbial fermentation by microorganisms in a cheap and more effective manner.
Objective
This study aims to isolate and characterize microorganisms that have the capability to produce vitamin B_{12}. In addition, the current work aims to optimize the vitamin B_{12} production conditions by isolating strains using suitable waste materials to obtain a high vitamin B_{12} yield.
Materials and methods
Different bacterial and yeast isolates were isolated from marine and food samples using the pour-plate technique. These isolates were screened for vitamin B_{12} production using a specific growth medium that lacked vitamin B_{12} and a test indicator bacterium. The content of vitamin B_{12} was estimated using spectrophotometer measurement and high-performance liquid chromatography (HPLC). The isolates that showed high vitamin B_{12} productivity were identified using MALDI-TOF technique. The identified strains were implemented for the optimization of vitamin B_{12} production to reveal the most proper and optimum conditions for the production. Response surface methodology (RSM) was employed to enhance the production of vitamin B_{12} in a flask scale. Agro-industrial wastes such as molasses were used for vitamin B_{12} production using the most optimum conditions as determined by RSM.
Results and conclusion
Eighty-seven actinomycetes, bacterial, and yeast isolates were screened for vitamin B_{12} production. Out of these isolates, 15 showed high vitamin B_{12} productivity. We found that bacilli and yeast isolates were the most productive among the tested cocci and actinomycetes isolates. The highly productive *Bacillus* and yeast isolates were identified using the MALDI-TOF analysis. The isolates were identified as *Candida pelliculosa*, *Geotrichum candidum*, *Bacillus subtilis* and *Bacillus sp*. One strain of *Candida pelliculosa* (coded BYI), three strains of *Geotrichum candidum* (coded as MZYC, MZYD, and MZYG) were selected for studying the effect of sugar type and inoculum size on the yield of vitamin B_{12} production. Strain MZYD was selected for the statistical modelling using RSM to optimize seven factors for the vitamin B_{12} production. These factors included temperature, fermentation time, salt concentration, pH, sugar concentration, inoculum size, and aeration. Five factors *i.e.*, temperature, pH, sugar concentration, and inoculum size were shown to significantly improve the vitamin B_{12} production. A maximum yield of 64.21 μg/100 ml was obtained using the optimized RSM conditions. These optimized conditions were used to produce vitamin B_{12} using molasses as a raw material for the microbial growth.

Keywords:
cyanocobalamin, methylcobalamin, vitamin B_{12}, vitamins

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Introduction

The term 'vitamin' is derived from the Latin words 'vital' and 'amine' because vitamins are required for life and were originally thought to be amines. Vitamins are essential micronutrients for the metabolism of all living organisms that cannot be synthesized by mammals. Most of the vitamins are synthesized by microorganisms and plants [1,2]. Vitamins are classified as either water-soluble vitamins (B-group and C) or fat-soluble vitamins (A, D, E, and K) [3].

Contrary to the most vitamins that are synthesized either chemically or by microorganisms, Vitamin B_{12} production is only confined to a few bacteria as it relies on microbial fermentation [4]. Vitamin B_{12} biosynthesis is very complicated, and it is very expensive to synthesize it chemically due to the complexity of the pathways included in its production [5,6].

Vitamin B_{12} has a complicated structure and properties compared with the other vitamins [7,8]. The main sources of vitamin B_{12} are the animal-based foods only such as red meat, milk, cheese, egg, fish, and shellfish [9]. Therefore, the current study was planned with a particular focus on the production of vitamin B_{12} as an essential and a unique vitamin.

Vitamin B_{12} was first isolated as cyan form in 1948 when Folkers and Smith were able to isolate the antipernicious anemia factor from liver extracts [10,11]. This was followed by further studies leading to the award of Nobel prize to five scientists (George Hoyot Whipple along with George Minot and William Murphy in 1934, Alexander Todd in 1957, and Dorothy Hodgkindue in 1964) for their direct and indirect efforts of vitamin B_{12} discovery [6,12,13].

Vitamin B_{12} ($C_{63}H_{88}CoN_{14}O_{14}P$) generic name is 'cobalamin' because of containing cobalt metal, which gives its red color [10]. It has four forms including methyl cobalamin, deoxy adenosyl cobalamin, hydroxyl cobalamin, and cyanocobalamin which is the most widely used form in supplements and prescription drugs [14]. Indeed, vitamin B_{12} cyanocobalamin form (CNCbl) is considered as 'provitamin'. It is not the direct active vitamin form in humans, it needs to be converted into the cofactors methyl cobalamin (MeCbl) and coenzyme B_{12} (adenosylcobalamin, AdoCbl), which is considered as the active vitamin B_{12} form [15].

Vitamin B_{12} plays a vital role in the immune system healthy balance and DNA synthesis. It has a leading role in the nervous system function that maintenance nerve cell and cooperating with cell synthesis in addition the catabolism of proteins and fatty acids [16,17].

The main source of vitamin B_{12} in human is animal-based foods [18]. Therefore, vegans and vegetarians are most likely to be affected by vitamin B_{12} deficiency [19]. While vitamin B_{12} is stored mainly in the liver, the body stores it until it is needed. When stopping consuming it, then eventually becomes deficient and diet cannot maintain the levels. Some factors lead to the depletion of hepatic stores and deficiency occurs as cases in which vitamin B_{12} cannot be absorbed due to dietary insufficiency including malabsorption, or lack of intrinsic factor (a glycoprotein required for its absorption). A deficiency of vitamin B_{12} causes pernicious anemia, Neurologic sequelae represented in paresthesia, demyelination of the corticospinal tract, dorsal columns, and peripheral neuropathy. In addition, the deficiency of vitamin B_{12} causes psychiatric disorders such as irritability, depression, impaired memory, and dementia [20]. Most cases of vitamin B_{12} deficiency can be easily treated with injections or tablets to replace the missing vitamin [21]. Several microorganisms were used to produce vitamin B_{12} such as, *Propionibacterium freudenreichii, Ralstonia eutropha* [22], *Candida sp* [23]. *Lactobacillus plantarum* [24]. *Propionibacterium shermanii* [25], *Bacillus megaterium* [26], *Propionibacterium spp* [27], *Klebsiella sp*., *Saccharomyces cerevisiae, Rhizopus oligosporus* [28], *Yarrowia lipolytica* [29].

The fermentation medium must be carefully optimized to maximize the productivity, as this affects cell growth and the expression of desired metabolites [30]. The optimization should be performed before the industrial-scale metabolite manufacturing. Numerous statistical and nonstatistical methods for medium optimization have been thoroughly investigated. One-factor-at-a-time (OFAT), a nonstatistical approach, reveals important parameters and their useful ranges. However, OFAT takes a lot of time and trials for several tests to demonstrate the impact of different parameters. Furthermore, it rarely considers the impact of multiple factors and their interactions at once, which is problematic [31]. Therefore, statistical experimental design techniques are needed to produce statistical models that simultaneously examine multiple independent variables and describe the link between variables [32]. Response surface methodology (RSM) is a statistical optimization technique that optimizes process yield by using experimental factorial designs, such as Box-Behnken and specifies the response's behavior in the selected design space [33,34]. The interaction between the components that have a substantial impact on product information is studied using Box-Behnken design. RSM uses the data from the Box-Behnken experimental runs to find the mathematical model that connects process parameters and outcome [35].

Consequently, this study aims to synthesize vitamin B_{12} via yeast and bacterial strains isolated from marine

and food sources in Egypt and optimize the vitamin B_{12} production using the RSM.

Materials and methods

Sampling

Different samples were collected from different places. Marine water samples were collected from El-Ein El-Sokhna, the Red Sea in Egypt. Lake water samples were collected from Ein al-Sira lake, Cairo, Egypt. Grapes, banana, barley, Juhayna rayeb samples were collected from different markets in Giza area. Soil samples were collected from El-Manial district, Cairo, Egypt.

Raw material

Egyptian sugarcane molasses was obtained from El-Hawamdia factory for the integrated sugar industry and clarified to be implemented as an agricultural waste material for vitamin B_{12} production via microorganisms being isolated in this study.

Microorganism

Lactobacillus leichmannii ATCC 7830 was used as a test and indicator organism for vitamin B_{12} production. This strain was obtained from microbial resource center, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

Isolation and screening of vitamin B_{12} producing isolates

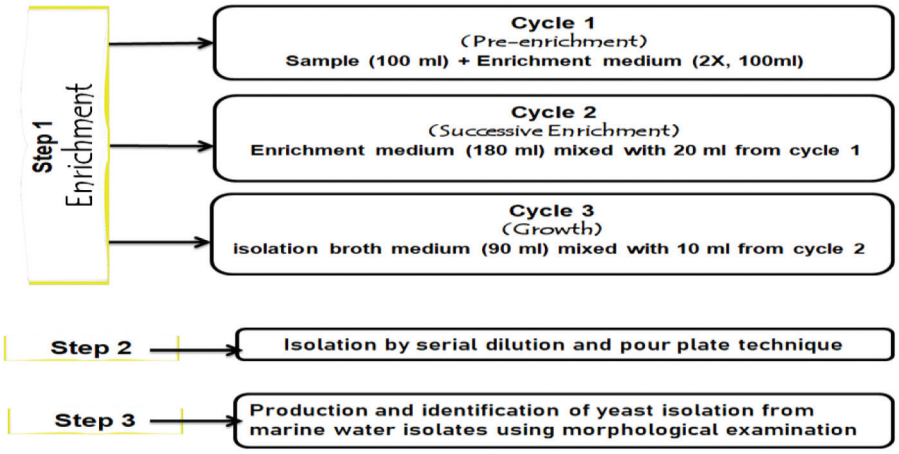
For yeast isolation from marine samples, a three-step method was applied for yeast enrichment and isolation as shown in Fig. 1 [36]. While yeasts from barley, banana, and grape samples were isolated on malt extract, glucose, yeast extract, peptone medium (MGYP) containing (g/l): malt extract 10; glucose 10; yeast extract 3; peptone 10. Plates were incubated at 25°C for 2–5 days [37].

For bacterial isolation from marine water and food samples, nutrient agar medium was used [38]. The bacteria were isolated using the pour plate technique. The cultures were incubated at 30°C for 24–48 h. Actinomycetes were isolated on Bennets agar medium [39].

Different bacterial and yeast isolates from different sources were examined for their potentials for vitamin B_{12} production. The isolates were screened for vitamin B_{12} by growing them on vitamin B_{12} assay medium at 30°C for 24–48 h adopting the methods described by [40,41]. The growth of isolates on this medium in the absence of vitamin B_{12} indicates their ability to synthesize vitamin B_{12}.

The isolates that showed growth on the medium were further tested to confirm their capabilities to produce vitamin B_{12} by using an auxotrophic indicator bacterial strain *(Lactobacillus leichmannii* ATCC 7830). This strain can only grow in the presence of vitamin B_{12}. Therefore, this strain was plated on vitamin B_{12}-free agar medium where two wells were punched in each plate using a sterile corkborer and filled with 100 μl of the supernatant of the selected isolates that are suspected to be vitamin B_{12} producers. For control samples, 100 µl of pure standard cyanocobalamin solution (LOBA CHEMIE PVT.LTD) (2-10 µg/ml) were placed in two wells per each plate. The cultures were incubated at 37°C for 24 h and examined for the developed growth zone around the wells [42,43].

Figure 1



Schematic diagram for the enrichment and isolation of yeasts.

Lactobacillus leichmannii ATCC 7830 was maintained and cultivated on MRS Broth (Lactobacillus MRS Broth) (TM MEDIA) at 37°C for 24 h. The strain culture was centrifuged, washed with saline, and suspended in 10 ml saline to be used as an indicator organism for vitamin B_{12} production.

The selected isolates that are confirmed to be vitamin B_{12} producers were morphologically characterized using the Gram stain method. The isolates were maintained as pure cultures at −80°C for further analysis.

Vitamin B_{12} production

Inoculum preparation

Erlenmeyer flasks containing 100 ml of sterile inoculum medium containing (g/l): peptone 5; yeast extract 3; glucose, 10; potassium di-hydrogen phosphate 2; tween-80 0.1 [44] were inoculated with the selected *Bacillus* and yeast isolates and allowed to grow for 24 h using shaker incubator (innova 4300) at 110 rpm.

For actinomycetes, Bennets broth medium was used for inoculum preparation containing (g/l): Yeast extract, 1; Beef extract 1; Casein enzymic hydrolysate 2; dextrose 10 and allowed to grow for 2–5 days [39].

Fermentation

A 5 ml of yeast and *Bacillus* isolates inocula were added to 100 ml of Zaky's production medium containing (g/100 ml): xylose 12; glucose 12; malt extract 1.2; yeast extract 1.2; peptone 2; $(NH_4)\ 2SO_4$ 0.4; KH_2PO_4 0.025; $COSO_4.7H_2O$ 1 [36].

For actinomycetes, a production medium containing (g/l): NH_4PO_4 2; KH_2PO_4 2; $CaCl_2.2H_2O$ 0.01; $FeSO_4.\ 7H_2O$ 0.005; $MnSO_4.nH_2O$ 0.005; $COSO_4.7H_2O$ 1; glucose 10 [45] was used.

Fermentation was carried out at 30°C for 2 days for yeast and *bacillus isolates*, whereas the fermentation process for actinomycetes was conducted for 5 days. At the end of fermentation, dry biomass of the vitamin B_{12}-producing isolates and vitamin B_{12} yield were determined (Fig. 2).

Biomass estimation

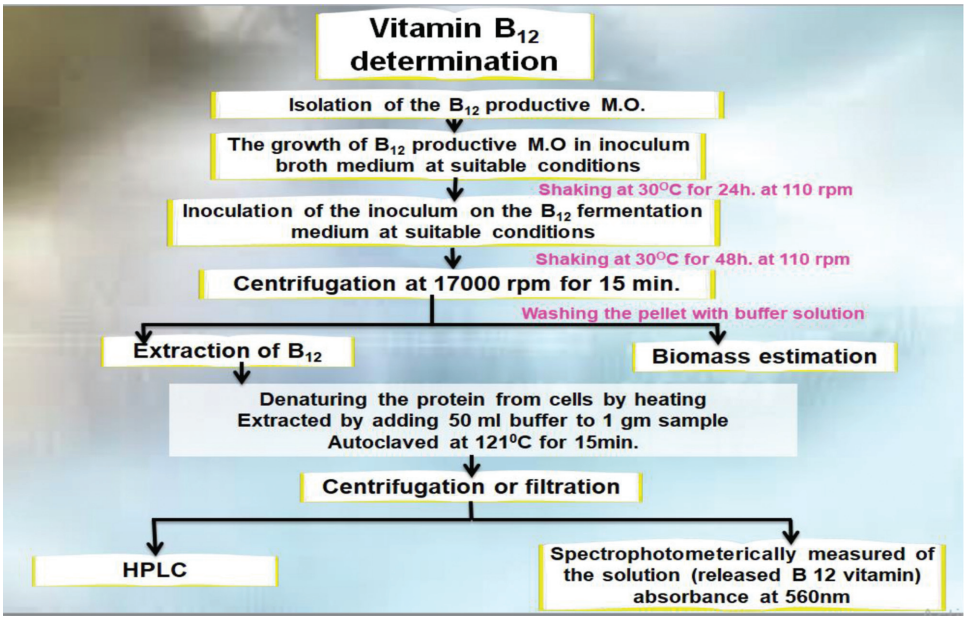
100 ml of the isolates cultures were centrifuged at 10 000 rpm for 10 min through HERMLE z323k centrifuge [39]. The pellet was then desiccated in an electric oven for drying at 80°C for 24 h [46].

Extraction and estimation of vitamin B_{12}

Vitamin B_{12} is bound to protein and must be disassociated before analysis. This is often accomplished simply by decomposing 1 gm of sample with 50 ml buffer solution and denaturing the protein by heating during the biomass estimation process.

The extraction of vitamin B_{12} was performed by harvesting the cells from fermentation broth via

Figure 2



Vitamin B12 determination workflow.

centrifugation of the suspensions at 17 000 rpm for 15 min [46,47]. The pellet was washed with sterile buffer solution (pH, 6) containing (g/100 ml distilled water): disodium hydrogen phosphate 1.29; citric acid 1.1; sodium metabisulfite 1; potassium cyanide 0.01. The supernatant containing extracted vitamin B_{12} was centrifuged to get an extracted solution of vitamin B_{12} without particles. The extracted solution containing vitamin B_{12} was spectrophotometrically measured on PG instruments Ct60 UV visible spectrophotometer at 560 nm [48].

Furthermore, the vitamin B_{12} yield was also estimated using high performance liquid chromatography (HPLC). HPLC analysis was carried out using injection of the purified sample on C-18 column with a PDA detector. The absorbance was measured at two wave lengths *i.e.,* 361 and 546 nm. The solvent A was 50% methanol with 0.1% acetic acid while the solvent B water, with a flow rate of 1 ml/min for 40 min. The retention times (from 0 to 90%) of vitamin B_{12} compound was recorded with a linear gradient of a methanol solution (50% v/v) [49,50].

Identification of vitamin B_{12} producing isolates using MALDI-TOF MS analysis

Selected vitamin B_{12}-producing isolates were subjected to MALDI-TOF MS analysis as described by McElvania and Burnham, 2014 and Schulthess *et al.*, 2016. Briefly, fresh 24–48 h cultures of the isolates were subjected to full protein extraction by emulsifying them in 50 μl of sterile water and 50 μl of 96% ethanol. This was followed by spotting one microliter of the suspension onto a 96-spot stainless steel target plate (Bruker Daltonics, Bremen, Germany) and air drying for 2 min. Dried spots were overlaid with 1 μl of 70% formic acid and 1 μl of HCCA matrix (Bruker Daltonics). Fully dried plates were subjected to MALDI-TOF MS analysis. The mass spectrum of the isolates was compared with the reference spectra database or library, producing a list of the most closely interrelated isolates with numeric rankings [51,52].

Hemolysis test on blood agar

To evaluate the pathogenicity and hemolytic properties of the identified strains, hemolysis test on blood agar plates was determined according to the protocol method described by [53]. The selected isolates were streaked on blood agar plates and incubated at 30°C for 24 h. The isolates that showed gamma (γ) or no hemolysis were considered safe or non-pathogenic.

Optimization of vitamin B_{12} production

The examined strains that showed no hemolysis and high vitamin B_{12} productivity were further used in fermentation processes for optimizing the optimum conditions for maximum B_{12} production. Optimization was carried out as OFAT to study the most suitable sugar type (glucose, sucrose, fructose, lactose, and galactose) and inoculum size (5, 10, and 15%) at constant conditions of temperature (30°C), fermentation time (2 days), salt concentration (0%), pH (6.5), sugar concentration (12%), and aeration (110 rpm).

Statistical modelling using response surface methodology

The most promising strain that can produce the highest yields of vitamin B_{12} was selected for optimization using RSM. In this experiment, the Box-Behnken design was applied to design fermentation experiments as a modeling technique. This design was implemented to assess the relationships between set of variables and observed results. The statistical analysis and modeling were performed using the Design-Expert v7.0.0 software (Stat- Ease, Inc. MN, USA). Sixty fermentation runs were designed based on the Box-Behnken design of seven factors—temperature, A (°C); fermentation time, B (days); salt concentration, C (%); pH, D; sucrose concentration, E (%); inoculum size, F (%); aeration, G (rpm). Each variable was coded at three levels (– 1, 0,+1) to describe the most optimum response surface. The responses produced from this experimental design were the vitamin B_{12} concentration and the biomass yield.

Optimization of the production medium with molasses

Clarified molasses was chosen as a waste material to represent the main sugar source in the production medium instead of glucose. The used molasses contains 9.5% glucose, 31% sucrose, 10% fructose, 0.95% nitrogen, and 80% total solids. Molasses clarification was conducted by adding concentrated H_2SO_4 (3 ml) to molasses (1 kg) mixed with distilled water (1 l), and the pH was adjusted to 3.5. This was followed by boiling the mixture for 30 min in a water bath. The mixture was completed to 2 L after cooling and keeping for overnight in the refrigerator. The mixture was then centrifuged and sterilized at 121°C for 15 min. Final sugar concentration of the clarified molasses was 25% [54]. Based on the results obtained in the statistical modeling, the sugar level of clarified molasses was adjusted to 6 and 12% and the pH was adjusted to 6.5 and 7.5 to attain the highest vitamin B_{12} production.

Molasses synthetic medium was prepared to contain all the components of the production medium as aforementioned [45] with the replacement of glucose with the clarified molasses.

Statistical analysis

The statistical modeling of vitamin B_{12} optimization and figures were performed using Design Expert software (version 13.0). GraphPad prism 10 software was used for the statistical analysis to calculate the standard error of the mean and the *P* value of validation data.

Results and discussion

Isolation and screening of vitamin B_{12}-producing isolates

Eighty-seven isolates were isolated from different samples and sources and used in the screening survey of vitamin B_{12}-producing microorganisms. The cellular morphologies of the different isolates are distinct as they are bacilli, cocci, oval, and actinomycetes as shown in (Table 1). Since yeasts that are present in marine environments survive high exogenous salt content, high concentration of inhibitory compounds, and low carbon and nitrogen levels, to overcome these conditions, a new method for yeast isolation from marine water samples was implemented using Zaky's Enrichment medium (Fig. 1) [36].

Table 1 Number of vitamin B_{12}-producing microorganisms isolates

Microbial groups	Total number of isolates	Number of vitamin B12-producing microorganisms
Bacilli	39	8
Cocci	21	1
Actinomycetes	5	1
Yeast	22	6
Total	87	16

The isolates codes, morphology, the amount of grown biomass, and the vitamin B_{12} yield are shown in (Table 2). Among the 87 isolates screened for vitamin B_{12} production, 12 isolates could grow on a vitamin B_{12}-free agar medium. They also showed a growth zone around the wells that were pre-inoculated with the indicator test organism *Lactobacillus leichmannii* ATCC 7830. This growth zone indicates that these isolates are vitamin B_{12}-producers. On the other hand, the other 75 isolates didn't show any growth in the vitamin B_{12}-free agar medium and growth zones around the wells that were pre-inoculated with the indicator test organism *Lactobacillus leichmannii* ATCC 7830. It was revealed that yeast isolates (BYI, MZYC, and BYB) exhibited the highest yield of vitamin B_{12} content (169.89, 163.88, and 127.76 μg/100 ml culture). In contrast, the *Bacillus* isolates (BYBK and MZB7) exhibited a lower yield of vitamin B_{12} content (95.65 and 9.59 μg/100 ml culture) compared with yeast isolates. Among the examined isolates, the most productive ones were *Bacillus* and yeast isolates. Therefore, *Bacillus* and yeast isolates were selected for further studies.

Vitamin B_{12} is existed in four forms (cyanocobalamin, methylcobalamin, adenosyl cobalamin, and hydroxycobalamin) [15]. MZYC and MZYD isolates were subjected to HPLC analysis to detect the levels of vitamin B_{12} in the form of cyanocobalamin and methylcobalamin.

The HPLC analysis was conducted for the vitamin B complex. Only vitamin B_{12} was detected in both isolates, and traces of vitamin B_5 were detected only

Table 2 Determination of vitamin B12 yield produced by the selected isolates

Isolate code	Group	Source	Biomass g/100 ml.	Vitamin B12 μg/100 ml culture	Vitamin B12 μg/g cells culture
GYA	yeast	Grapes	0.5±0.02	51.95±0.06	103.9
BYB	yeast	Banana	0.8±0.02	127.76±0.10	159.7
MZYC	yeast	Marine	0.1±0.00	163.88±0.18	1638.8
MZYD	yeast	Marine	0.5±0.02	65.55±0.07	131.1
MZYG	yeast	marine	0.6±0.02	82.27±0.05	137.12
BYI	yeast	Barley	0.1±0.00	169.89±0.15	1698.9
BYBK	Gram+ bacilli	Soil	0.2±0.01	95.65±0.07	478.25
SYC6	Gram+ Streptococci	Soil	0.5±0.01	104.79±0.07	209.58
LTBp (1-5)	Gram+ bacilli	Lake	0.09±0.00	0.89±0.00	9.89
LTBr51	Gram+ bacilli	Lake	0.46±0.02	5.35±0.01	11.63
MZB7	Gram+ bacilli	Marine	0.6±0.02	9.59±0.01	15.98
JTAb	Gram+ Actinomycetes	Juhayna Rayeb	0.1±0.00	10.60±0.03	106

Each value represents a mean of three replicates±standard error of the mean. Determination conditions: 12% glucose as a type of sugar, 10% inoculum size, 30°C, and aeration rate of 110 rpm.

in MYZC isolate. It was revealed that both isolates produce vitamin B_{12} in the form of cyanocobalamin and methylcobalamin. MZYC Isolate showed a higher yield of cyanocobalamin than MZYD isolate (244.29 and 80 mg/gm). While MZYD isolate showed a higher yield of methylcobalamin than MZYC isolate (223.52 and 46.57 mg/gm) (Fig. 3). Methylcobalamin was detected in MZYD and MZYC isolates at retention times ranged from 4.837 to 5.013. These findings are in agreement with Veronica and Sumathy study who showed that methylcobalamin was detected in *Streptomyces spp* strains at retention time ranged from 2.669 to 5.614 [55].

Identification of the vitamin B_{12}-producing isolates

Seven yeast and bacterial isolates producing vitamin B_{12} that showed high productivity were selected for identification by MALDI-TOF MS technique. The identity and similarity % of the identified strains are listed in (Table 3) and the morphological characteristics of the identified strains are shown in (Fig. 4).

Pathogenicity and hemolysis of identified strains

All seven identified strains were grown on blood agar medium to test their hemolysis. The hemolysis results are listed in (Table 4 and Fig. 5). All strains showed gamma-hemolysis (nonhemolytic), which indicates their nonpathogenicity nature. The hemolysis is classified into three types: Gamm (γ), Alpha (α), and Beta (β) hemolysis. If there was no reactions or changes on the medium it means gamma hemolysis, while the brown or green shades surrounding the colonies indicated an alpha hemolysis. Clear or transparent zones surrounding the colonies refers to beta hemolysis which showed the capability of strain to destroy and lysis the red blood cells [52].

Optimization of vitamin B_{12} production

Four yeast strains (MZYC, MZYG, MZYD, and BYI) were selected for studying the optimum conditions for vitamin B_{12} production as OFAT including constant conditions of temperature (30°C), fermentation time (2 days), salt concentration (0%), pH (6.5), and aeration (110 rpm). Additionally, the production was performed under varying conditions of sugar type (glucose, sucrose, fructose, lactose, and galactose) and inoculum size (5, 10, and 15%) and to select the most optimum sugar type and inoculum size.

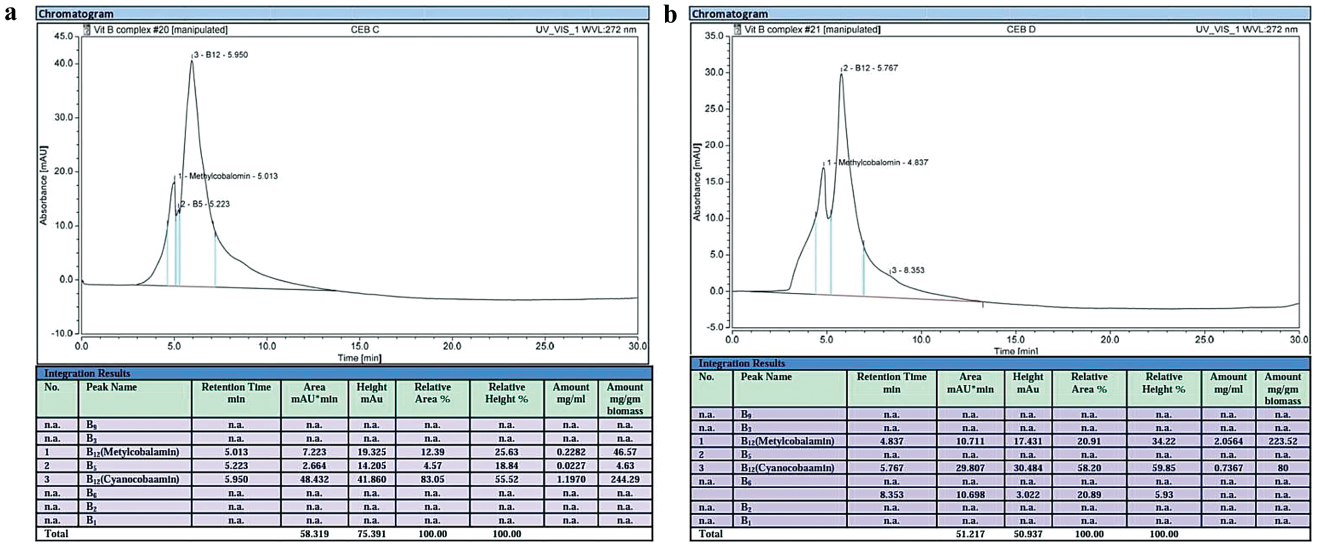
The effect of sugar type on vitamin B_{12} production

Data presented in (Table 5) show that sucrose and glucose are the most suitable carbon sources for vitamin B_{12} production by the strain (MZYC). This finding indicates the magnitude of using industrial wastes such

Table 3 Identification of vitamin B_{12} producing isolates by MALDI-TOF MS technique

Isolate code	Yeast or bacterial identification	%similarity % confidence
GYA	*Candida pelliculosa*	99.21
BYB	*Candida pelliculosa*	99.17
MZYC	*Geotrichum candidum*	99.25
MZYD	*Geotrichum candidum*	99.46
MZYG	*Geotrichum candidum*	99.08
BYI	*Candida pelliculosa*	99.16
BYBK	*Bacillus subtilis*	98.79

Figure 3



Integration Results

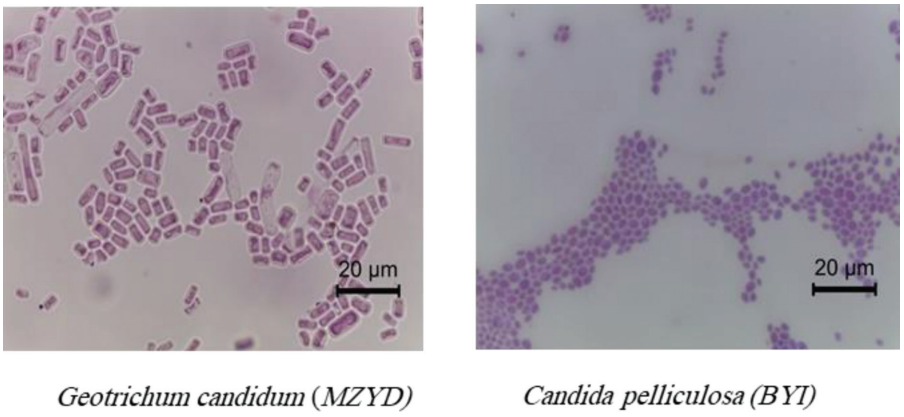
No.	Peak Name	Retention Time min	Area mAU*min	Height mAu	Relative Area %	Relative Height %	Amount mg/ml	Amount mg/gm biomass
n.a.	B_9	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	B_3	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
1	B_{12}(Metylcobalamin)	5.013	7.223	19.325	12.39	25.63	0.2282	46.57
2	B_5	5.223	2.664	14.205	4.57	18.84	0.0227	4.63
3	B_{12}(Cyanocobaamin)	5.950	48.432	41.860	83.05	55.52	1.1970	244.29
n.a.	B_6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	B_2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	B_1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Total			58.319	75.391	100.00	100.00		

Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAu	Relative Area %	Relative Height %	Amount mg/ml	Amount mg/gm biomass
n.a.	B_9	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	B_3	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
1	B_{12}(Metylcobalamin)	4.837	10.711	17.431	20.91	34.22	2.0564	223.52
2	B_5	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
3	B_{12}(Cyanocobaamin)	5.767	29.807	30.484	58.20	59.85	0.7367	80
n.a.	B_6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
		8.353	10.698	3.022	20.89	5.93	n.a.	n.a.
n.a.	B_2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	B_1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Total			51.217	50.937	100.00	100.00		

High performance liquid chromatography chromatogram of vitamin B12 showing peaks for a. Strain MZYC and b. Strain MZYD. Vitamin B12 detected as cyanocobalamin and methylcobalamin.

Figure 4



Cellular morphological shapes of the identified strains using Gram stain and microscopic examination.

Table 4 Pathogenicity of the identified strains as indicated by the hemolysis test

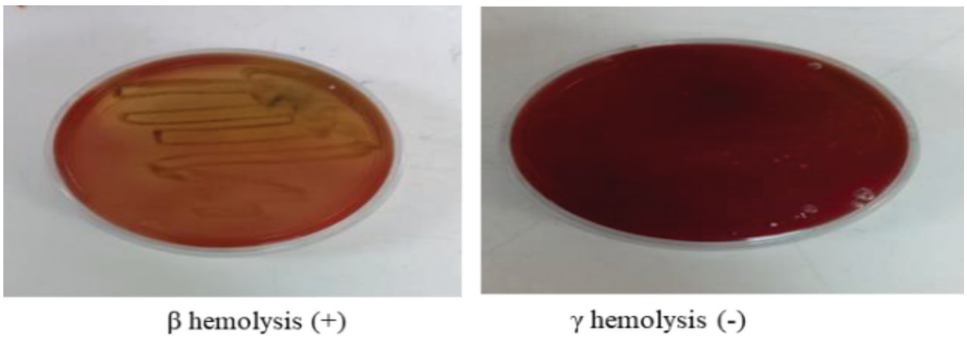
Strian code	Strains	Hemolysis results
GYA	*Candida pelliculosa*	γ hemolysis (–)
BYB	*Candida pelliculosa*	γ hemolysis (–)
MZYC	*Geotrichum candidum*	γ hemolysis (–)
MZYD	*Geotrichum candidum*	γ hemolysis (–)
MZYG	*Geotrichum candidum*	γ hemolysis (–)
BYI	*Candida pelliculosa*	γ hemolysis (–)
BYBK	*Bacillus subtilis*	γ hemolysis (–)

as molasses as raw materials for vitamin B_{12} via microbial fermentation. On the other hand, the strain (MZYG) showed an optimum vitamin B_{12} yield with galactose (12.93 µg/100 ml culture). Strain MZYD produced notable yield of vitamin B_{12} when using sucrose and lactose as carbon sources (23.41 and 17.17 µg/100 ml culture) compared with the produced amount when using glucose and fructose (3.79 and 5.35 µg/100 ml culture) as carbon sources.

Strain BYI exhibited the lowest productivity of vitamin B_{12} (0.67 µg/100 ml culture) when using lactose as a carbon source. Therefore, using whey in vitamin B_{12} production via microbial fermentation isn't preferred for this strain. Similar studies on the production of vitamin B_{12} from yeasts showed that yeasts can produce vitamin B_{12}. Grygier *et al*., 2019 showed that strain *Galactomyces geotrichum 38* could produce vitamin B_{12} with a yield of (92 µg/L) at the onset of its growth and increased to (224 µg/l) after 216 h [56]. Additionally, García *et al*., 2014 reported that *Kluyveromyces marxianus* and *Candida utilis* produce vitamin B_{12} with a yield of 0.0001-0.015–0.05 µg g^{-1} [57].

Wang and colleagues studied the effect of using glucose and glycerol on vitamin B_{12} production from *Propionibacterium freudenreichii*. He showed that using glucose enhanced the production and resulted in more productivity of vitamin B_{12} (0.35 mg/l h). However, the glycerol was shown to produce lower amount of vitamin B_{12} (0.14 mg/l h). While applying a co-fermentation process by adding both glucose and glycerol increased the productivity (biomass 0.68 mg/g) and vitamin B_{12} yield (0.37 mg/l h) [58].

Figure 5



The pathogenicity and hemolysis patterns of the identified strains using blood agar plates.

Table 5 The effect of sugar type on the production of vitamin B_{12}

Strain	Sucrose			Glucose			Fructose			Galactose			Lactose		
	Biomass g/100 ml	Vitamin B12 µg/100 ml culture	Vitamin B12 µg g-1cells culture	Biomass g/100 ml	Vitamin B12 µg/100 ml culture	Vitamin B12 µg g-1cells culture	Biomass g/100 ml	Vitamin B12 µg/100 ml culture	Vitamin B12 µg g-1cells culture	Biomass g/100 ml	Vitamin B12 µg/100 ml culture	Vitamin B12 µg g-1cells culture	Biomass g/100 ml	Vitamin B12 µg/100 ml culture	Vitamin B12 µg g-1cells culture
MZYC	0.9±0.01	9.14±0.00	10.16	0.8±0.03	8.25±0.03	9.52	0.4±0.02	4.24±0.00	10.59	0.6±0.02	6.91±0.02	11.52	0.2±0.01	5.57±0.02	27.87
MZYG	0.8±0.02	2.45±0.03	3.06	0.4±0.02	6.46±0.01	13.85	0.5±0.02	5.13±0.01	9.615	0.7±0.01	12.93±0.03	17.63	0.2±0.03	3.79±0.05	18.95
MZYD	0.9±0.00	23.41±0.01	26.01	0.6±0.02	3.79±0.02	6.32	0.6±0.02	5.35±0.01	8.92	0.3±0.03	9.81±0.04	29.43	0.8±0.01	17.17±0.01	21.46
BYI	0.63 ±0.06	13.38±0.01	21.40	1.11 ±0.07	6.35±0.02	5.72	0.62 ±0.00	11.93	19.24 ±0.03	N.D	N.D	N.D	1.29 ±0.00	0.67±0.00	0.52

Each value represents a mean of three replicates±standard error of the mean. Determination conditions:12% sugar type as a carbon source, 5% inoculum size, 30°C, and 110 rpm.

The effect of different sugar types on the vitamin B_{12} productivity via yeast strains is shown in (Fig. 6). According to these results and previous studies, the type of sugar was shown to have an essential impact on the vitamin B_{12} production. Hence selecting the suitable waste material is crucial for obtaining high yield of vitamin B_{12}.

Effect of inoculum size on vitamin B_{12} production by microbial strains

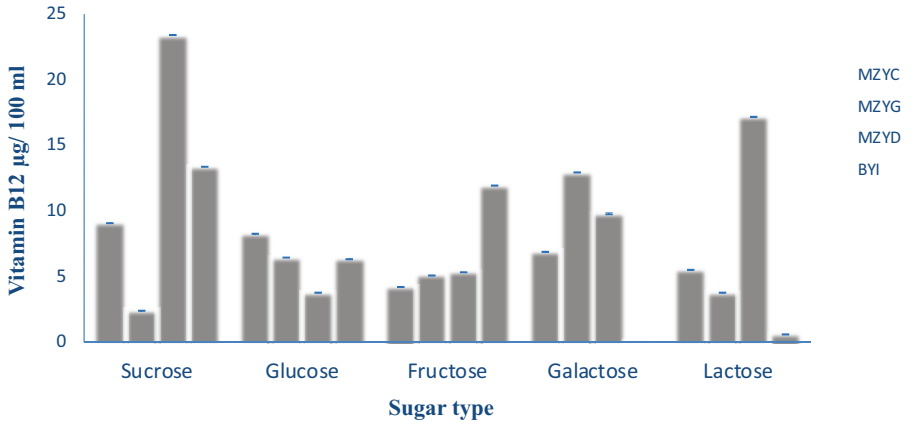
The inoculum size was shown to affect the vitamin B_{12} production. The vitamin B_{12} yield was significantly increased when increasing the inoculum size from 5 to 10% (Table 6). While increasing the inoculum size from 10 to 15% reduced the yield of vitamin B_{12}. Obtained results in Table 6 showed strain MZYD Biomass and production of vitamin B12 at 5, 10, 15% inoculum size (0.6, 0.5, and 1.93 g/100 ml) (3.79, 65.55, and 6.24 µg/100 ml of vitamin B_{12}). It is noticeable that increasing inoculum size from 5% to 10% significantly increased vitamin B_{12} production, while increasing the inoculum size to 15% reduced the production. It has been reported previously that supplementing the dietary meal for fish with the *Geotrichum Candidum* strain (10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11} CFU/kg) has improved the growth rate of fish only at 10^6–10^8 CFU/kg diet [59]. These results indicate that increasing the inoculum size would increase the competition between cells of *Geotrichum Candidum* and consequently reducing the growth rate. Similarly, our findings indicate that lower inoculum size increases the production of vitamin B_{12} probably due to better growth rate and efficient rates of nutrient consumption.

Statistical modelling using response surface methodology

Box-Behnken design was used to determine the most optimum conditions for vitamin B_{12} production and the impact of interactions among parameters. Temperature, pH, aeration, fermentation time, inoculum size, salt and sucrose levels were used as the parameters for the optimization using RSM.

The experimental results of the vitamin B_{12} amount (µg/100 ml) and the runs parameters are shown in (Table 7). The results revealed that the highest vitamin B_{12} concentrations (64.21 µg/100 ml) was obtained when the temperature, pH, aeration, fermentation time, inoculum size, salt and sucrose levels were 35°C, 6.5, 200 rpm, 2 days, 10% (v/v), 0, and 12% (g/v) respectively. A minimum vitamin B_{12} concentrations of 0.56 µg/100 ml was obtained when the temperature, pH, aeration, fermentation time, inoculum size, salt and sucrose levels were 30°C,

Figure 6



The effect of sugar type on the vitamin B12 productivity via the selected strains. Data are represented as the means of three replicate values ±standard error of the mean.

Table 6 Effect of inoculum size on strains for vitamin B_{12} production using glucose

Strain	5% inoculum			10% inoculum			15% inoculum		
	Biomass g/100 ml.	Vitamin B12 µg/ 100 ml culture	Vitamin B12 µg g^{-1} cells	Biomass g/100 ml.	Vitamin B12 µg/ 100 ml culture	Vitamin B12 µg g^{-1} cells	Biomass g/100 ml.	Vitamin B12 µg/ 100 ml culture	Vitamin B12 µg g^{-1} cells
MZYC	0.9±0.04	8.25±0.02	9.52	0.1±0.00	163.88±0.18*	1638.8	0.21±0.01	4.12±0.00	19.62
MZYD	0.6±0.00	3.79±0.00	6.32	0.5±0.02	65.55±0.07	131.1	1.93±1.25	6.24±0.02	3.23
MZYG	0.5±0.04	6.47±0.01	13.86	0.6±0.02	82.27±0.05**	137.12	1.005 ±0.39	1.67±0.00	1.67
BYI	1.11±0.07	6.35±0.00	5.72	0.1±0.00	169.89±0.15*	1698.9	1.13±0.00	2.12±0.00	1.88

Each value represents a mean of three replicates±standard error of the mean. ***P* less than 0.05, *P* less than 0.01. Determination conditions: 12% glucose as a carbon source at 30°C, and 110 rpm.

6.5, 110 rpm, 2 days, 5% (v/v), 0, and 12% (g/v) respectively.

For each parameter, Fig. 7 illustrates the impact of three values per each variable on the yield of vitamin B_{12}. The three values (-1, 0, and 1) represent the lowest, midpoint, and highest values in (Table 8). The temperature was shown to significantly affect the levels of vitamin B_{12} production ($P<0.0001$) at 35 °C (Fig. 7a). Similarly, pH, sucrose concentration, inoculum size and aeration significantly enhance the production levels of vitamin B_{12} at 7.5, 12%, 10%, and 200 rpm respectively (Fig. 7b–g).

Table 9 shows the ANNOVA analysis of response-surface cubic regression model where the F test and the corresponding *P* values were estimated. The effect of each variable was considered significant when the probability value, $P<0.05$. The determination coefficient of the regression equation R2 was equal to 0.9011. Thus, this model was shown to be adequate and can accurately predict the response as shown in Fig. 8. The model was validated by comparing the predicted values and actual experimental values and calculating the residual. The percentage error of the differences between the actual and predicted values for vitamin B_{12} yield was 0.31%.

The interactive effects of two variables on the vitamin B_{12} levels were analyzed. The response effect of interactions between temperature and other parameters was plotted as shown in Fig. 9. Figure 9a shows the effect of temperature and fermentation time on the vitamin B_{12} yield. The production of vitamin B_{12} was maximized at 2 days of the fermentation and at a temperature of 35°C. Consistently, the highest yield of vitamin B_{12} was obtained at a salt and sucrose concentration of 2% and 10% respectively with a temperature of 35°C (Fig. 9b and d). The production of vitamin B_{12} increased when the temperature increased to 35°C, whereas the vitamin B_{12} declined when the pH was lower than 5.5 (Fig. 9c). In addition, a combination of sucrose levels at 6% and a temperature of 35°C enhanced the vitamin B_{12} production (Fig. 9d). Moreover, the vitamin B_{12} yield increased at an inoculum size of 10% and aeration rate of 200 rpm (Fig. 9e and f). The ANNOVA analysis of most interactions showed a

Table 7 The experimental design matrix of the variables involved in the modeling using response surface methodology, and the obtained responses represented as biomass and vitamin B_{12} yield produced via *Geotrichum candidum* MZYD strain under different conditions

Std	Run	A	B	C	D	E	F	G	Biomass g/100 ml.	Vitamin B12 µg/100 ml culture	Vitamin B12 µg g-1cells
10	1	1	0	0	0	0	−1	−1	0.94	6.35	6.76
41	2	−1	0	−1	0	−1	0	0	0.73	11.59	15.88
18	3	0	1	0	0	−1	0	−1	0.79	0.67	0.85
25	4	−1	−1	0	−1	0	0	0	1.38	8.81	6.38
39	5	0	0	−1	1	0	0	1	0.3	12.04	40.13
54	6	0	1	−1	0	0	1	0	0.37	4.12	11.14
24	7	0	1	0	0	1	0	1	1.5	4.91	3.27
29	8	−1	−1	0	1	0	0	0	2.65	2.23	0.84
36	9	0	0	1	1	0	0	−1	0.91	1.34	1.47
53	10	0	−1	−1	0	0	1	0	1.37	8.03	5.86
19	11	0	−1	0	0	1	0	−1	1.02	1.67	1.64
26	12	1	−1	0	−1	0	0	0	0.62	6.47	10.44
7	13	0	0	0	−1	1	1	0	0.44	10.93	24.84
50	14	0	1	−1	0	0	−1	0	0.43	24.75	57.56
40	15	0	0	1	1	0	0	1	0.3	6.13	20.43
2	16	0	0	0	1	−1	−1	0	0.87	50.06	57.54
13	17	−1	0	0	0	0	−1	1	1.24	4.12	3.32
60	18	0	0	0	0	0	0	0	0.91	1.56	1.71
43	19	−1	0	1	0	−1	0	0	0.88	6.35	7.22
11	20	−1	0	0	0	0	1	−1	0.43	4.91	11.42
4	21	0	0	0	1	1	−1	0	0.68	31.86	46.85
56	22	0	1	1	0	0	1	0	0.3	5.24	17.47
23	23	0	−1	0	0	1	0	1	0.7	3.23	4.61
46	24	1	0	−1	0	1	0	0	0.42	2.01	4.79
31	25	−1	1	0	1	0	0	0	0.72	4.35	6.04
37	26	0	0	−1	−1	0	0	1	0.33	2.99	9.06
59	27	0	0	0	0	0	0	0	0.63	0.56	0.89
27	28	−1	1	0	−1	0	0	0	0.66	53.07	80.41
58	29	0	0	0	0	0	0	0	1.16	1.78	1.53
1	30	0	0	0	−1	−1	−1	0	0.74	2.12	2.86
30	31	1	−1	0	1	0	0	0	0.37	3.79	10.24
16	32	1	0	0	0	0	1	1	0.68	37.46	55.09
47	33	−1	0	1	0	1	0	0	0.48	1.45	3.02
15	34	−1	0	0	0	0	1	1	0.91	38.35	42.14
48	35	1	0	1	0	1	0	0	0.28	4.01	14.32
12	36	1	0	0	0	0	1	−1	0.91	42.7	46.92
17	37	0	−1	0	0	−1	0	−1	1.08	1.23	1.14
14	38	1	0	0	0	0	−1	1	0.94	64.21	68.31
44	39	1	0	1	0	−1	0	0	0.33	4.91	14.88
45	40	−1	0	−1	0	1	0	0	0.64	9.14	14.28
20	41	0	1	0	0	1	0	−1	0.47	1.34	2.85
28	42	1	1	0	−1	0	0	0	0.72	3.01	4.18
3	43	0	0	0	−1	1	−1	0	0.54	2.45	4.54
32	44	1	1	0	1	0	0	0	0.61	6.58	10.79
22	45	0	1	0	0	−1	0	1	0.76	2.79	3.67
6	46	0	0	0	1	−1	1	0	1.71	8.03	4.7
57	47	0	0	0	0	0	0	0	1.7	1	0.59
8	48	0	0	0	1	1	1	0	1.71	9.14	5.35
21	49	0	−1	0	0	−1	0	1	0.58	2.99	5.16
49	50	0	−1	−1	0	0	−1	0	1.73	2.99	1.73
51	51	0	−1	1	0	0	−1	0	1.19	0.89	0.75
35	52	0	0	−1	1	0	0	−1	0.5	10.14	20.28
38	53	0	0	1	−1	0	0	1	0.34	7.02	20.65

(*Continued*)

Table 7 (*Continued*)

Std	Run	A	B	C	D	E	F	G	Biomass g/100 ml.	Vitamin B12 µg/100 ml culture	Vitamin B12 µg g-1cells
52	54	0	1	1	0	0	−1	0	1.39	7.92	5.7
9	55	−1	0	0	0	0	−1	−1	0.58	33.78	58.24
55	56	0	−1	1	0	0	1	0	0.95	1	1.05
5	57	0	0	0	−1	−1	1	0	0.79	8.14	10.3
42	58	1	0	−1	0	−1	0	0	0.3	3.79	12.63
34	59	0	0	1	−1	0	0	−1	0.84	8.47	10.08
33	60	0	0	−1	−1	0	0	−1	0.77	0.67	0.87
Symbol		Levels									
				Factor					−1	0	1
A				Temperature (°C)					25	30	35
B				Fermentation time (days)					1	2	4
C				Salt concentration %					0	2	4
D				pH					5.5	6.5	7.5
E				Sucrose concentration %					6	12	18
F				Inoculum size (%)					10	12.5	15
G				Aeration (rpm)					0 (static)	110	200

significant effect on the vitamin B_{12} production ($P<0.05$) (Table 9). This indicates the effectiveness of these interactions for the vitamin B_{12} production.

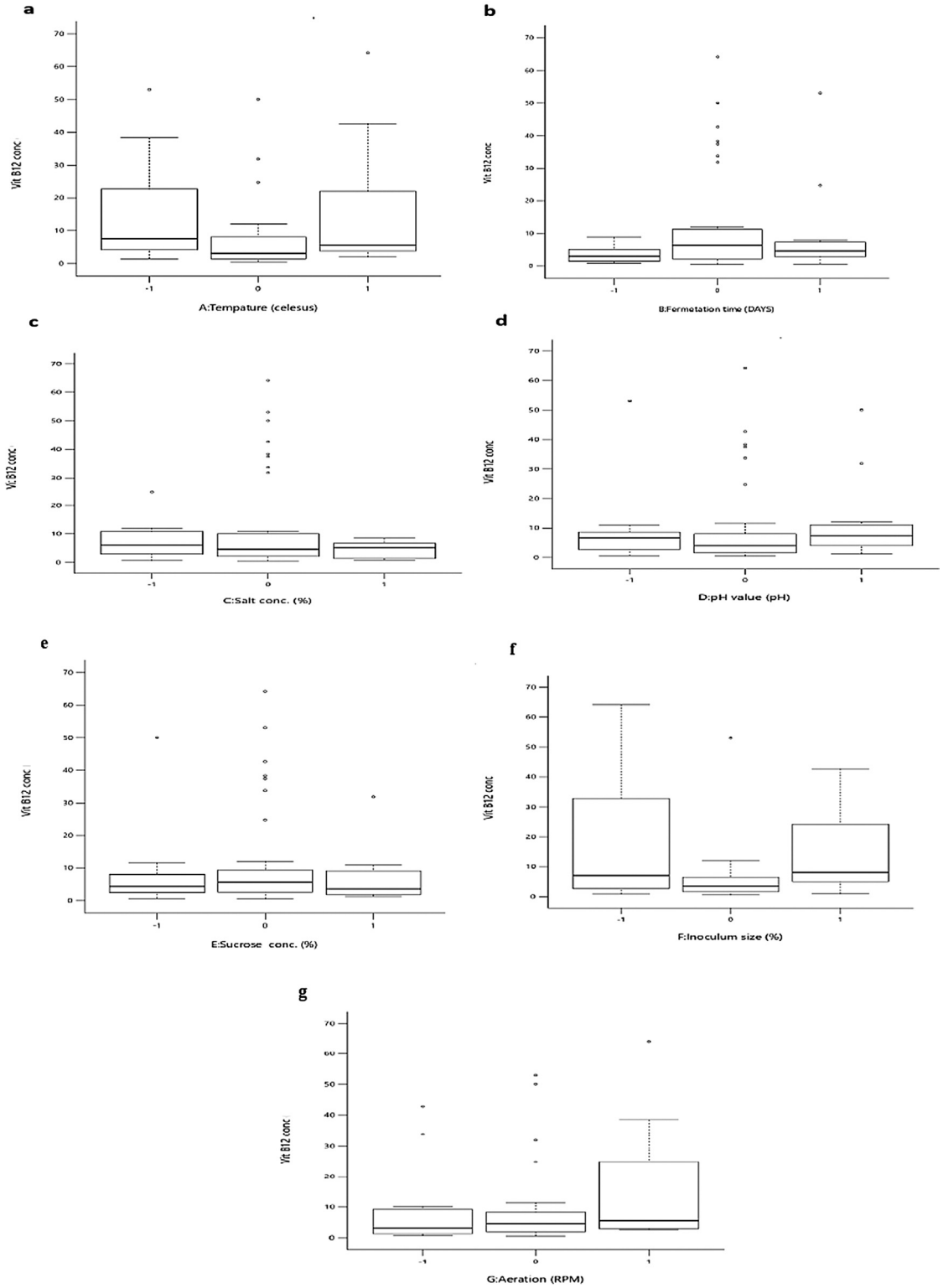
Figure 10 shows the 3D surface response of the interaction effects between different parameters on the vitamin B_{12} yield. The vitamin B_{12} yield increased at a higher temperature (>30°C) and a fermentation time of 2 days, given that the other parameters such as salt concentration, pH, sucrose concentration, inoculum size, and aeration were adjusted to 2, 5.42, 12, 10%, and 200 rpm, respectively as shown in Fig. 9a. This is in line with Wang *et al*., 2014 which reported that when adding glycerol to the medium of vitamin B_{12} production, the biomass (0.68±0.15, 0.68±0.12, and 0.72±0.08 mg/g) and vitamin B_{12} productivity (0.34±0.07, 0.34±0.12, and 0.36±0.07 mg/l h) increased at 12, 24, and 48 h while at 72 h, the growth (0.59±0.05, mg/g) and vitamin B_{12} productivity decreased (0.29±0.05 mg/l h). This could be a result of the presence of cell population growth in lag phase at the beginning of fermentation at 12, 24, and 48 h while the cell growth was in the decline phase at 72 h [58].

At a temperature higher than 35°C and salt concentration lower than 2%, the vitamin B_{12} production increased, given that the other parameters such as the fermentation time, pH, sucrose concentration, inoculum size, and aeration were adjusted to 2 days, 7.5, 6%, 10%, and 200 rpm respectively (Fig. 10b). This is in agreement with the results of Marcellino *et al*., 2001, who reported that *Getrichum candidum* cannot tolerate high salt levels as salting limited the growth of *Getrichum candidum* in dairy industry. It was shown previously that 1% NaCl resulted in a slight extinction of *Getrichum candidum* growth, and 5-6% NaCl caused an inhibitory effect [60]. This indicates that salting could limit the growth of *Getrichum candidum* and decrease the levels of vitamin B_{12} production.

The vitamin B_{12} yield increased when increasing the temperature from 30 to 35°C and increasing the pH from 5.5 to 6.5, given that the other parameters such as the fermentation time, salt concentration, sucrose concentration, inoculum size, and aeration were adjusted to 2 days, 2%, 6%, 10%, and 200 rpm, respectively (Fig. 10c). In the current study, it is noticeable that the highest yield of vitamin B_{12} was obtained at a pH around 6.5-7.5 while decreasing the pH value to 5.5 reduced the production. Khosravi *et al*., 2019 reported that *Propionibacterium freudenreichii* showed growth at a pH less than 4.5 while at pH values of 6.5–8.5, the vitamin B_{12} productivity was maximized [61].

The vitamin B_{12} yield increased when sucrose concentration increased from 6 to 12% and at a higher temperature (>30°C), given that the other parameters such as the fermentation time, salt concentration, pH, inoculum size, and aeration were adjusted to 2 days, 2%, 7.5, 10%, and 200 rpm respectively (Fig. 10d). Similarly, Fig. 9e and f show that the vitamin B_{12} production increased with increasing the temperature up to 35°C and the aeration up to 200 rpm. While the vitamin B_{12} production decreased with increasing the inoculum size more than 10%.

Figure 7



Box and whisker plots representing the mean for the effect of each single parameter on the vitamin B12 yield. a. The response effect of temperature. b. The response effect of fermentation time. c. The response effect of salt concentration. d. The response effect of pH. e. The response effect of sucrose concentration. f. The response effect of inoculum size. g. The response effect of aeration.

Optimization of the production medium with molasses

Data shown in Table 10 represent the effect of using molasses as a production medium providing the carbon source as sucrose for the optimization of vitamin B_{12} production conditions. The results showed that the pH value of 6.5 and 12% molasses were the best optimal conditions of vitamin B_{12} production via *Geotrichum candidum* MZYD strain producing the highest yield

Table 8 The experimental code of parameters involved in the modeling using response surface methodology with their coded and actual levels

		Levels		
Symbol	Factor	−1	0	1
A	Temperature (°C)	25	30	35
B	Fermentation time (days)	1	2	4
C	Salt concentration (%)	0	2	4
D	pH	5.5	6.5	7.5
E	Sucrose concentration (%)	6	12	18
F	Inoculum size (%)	10	15	20
G	Aeration (rpm)	(static) 0	110	200

(14.31 μg/100 ml) of vitamin B_{12}. The production was significantly higher compared with using molasses 6% as a production media (Table 10). While applying the same concentration (12% molasses) with increasing the pH to 7.5 led to a reduction in the yield of vitamin B_{12} to 10.55 μg/100 ml. However, reducing the pH in molasses to 6.5 led to a significant increase in the vitamin B_{12} concentration. On the other hand, reducing the molasses concentration to 6% led to a significant decrease of vitamin B_{12} production yielded vitamin B_{12} concentration of 3.49 and 5.57 μg/100 ml at both pH values of 6.5 and 7.5, respectively.

Table 9 Statistical analysis of variance (ANNOVA) of Box-Behnken design for all parameters affecting the vitamin B_{12} production using *Geotrichum candidum* MZYD strain

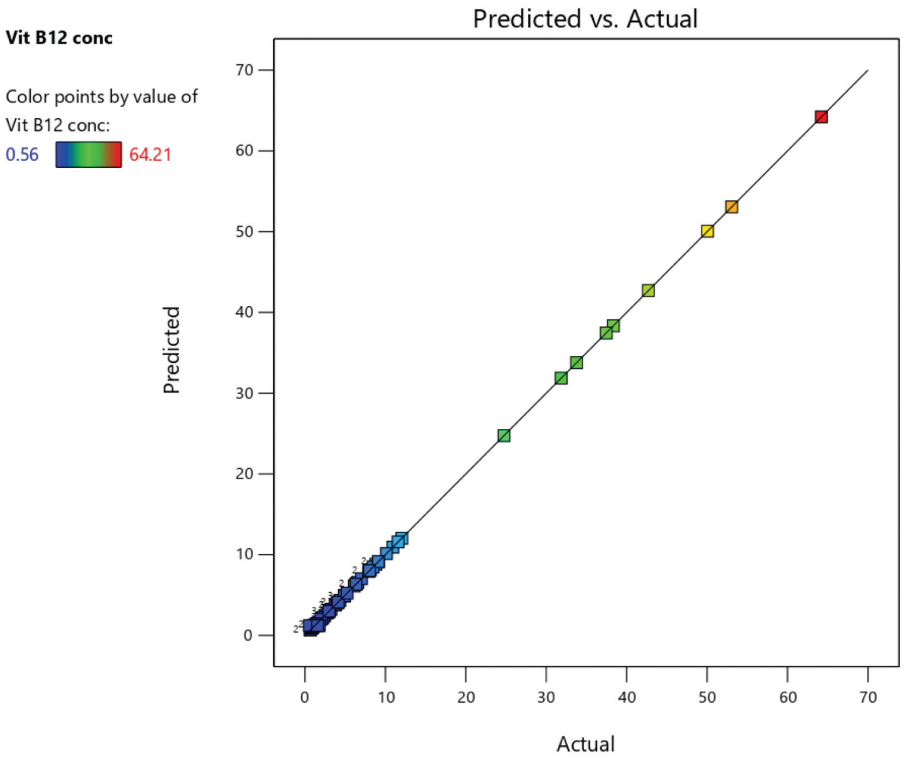
Source	Sum of squares	df	Mean square	F-value	P-value
Model	12253.8	56	218.82	718.93	>0.0001
A-Tempature	604.82	1	604.82	1987.16	>0.0001
B-Fermetation time	0.0435	1	0.0435	0.143	0.7305
C-Salt conc.	1.04	1	1.04	3.41	0.1621
D-pH value	711.59	1	711.59	2337.93	>0.0001
E-Glucose conc.	24.4	1	24.4	80.15	0.0029
F-Inoculum size	315.63	1	315.63	1037.02	>0.0001
G-Aeration	7.14	1	7.14	23.47	0.0168
AB	276.71	1	276.71	909.14	>0.0001
AC	32.2	1	32.2	105.79	0.002
AD	394.66	1	394.66	1296.67	>0.0001
AE	2.73	1	2.73	8.96	0.058
AF	2.25	1	2.25	7.38	0.0727
AG	298.17	1	298.17	979.63	>0.0001
BC	5.41	1	5.41	17.78	0.0244
BD	161.01	1	161.01	529.01	0.0002
BE	0.5565	1	0.5565	1.83	0.2692
BF	101.25	1	101.25	332.65	0.0004
BG	0.7021	1	0.7021	2.31	0.2261
CD	88.05	1	88.05	289.28	0.0004
CE	0.3081	1	0.3081	1.01	0.3885
CF	21.19	1	21.19	69.62	0.0036
CG	0.0968	1	0.0968	0.318	0.6122
DE	51.06	1	51.06	167.74	0.001
DF	785.07	1	785.07	2579.36	>0.0001
DG	4.23	1	4.23	13.91	0.0336
EF	59.24	1	59.24	194.64	0.0008
EG	0.1953	1	0.1953	0.6417	0.4817
FG	0	1	0	0	1
A^2	1111.59	1	1111.59	3652.15	>0.0001
B^2	105.17	1	105.17	345.53	0.0003
C^2	182.85	1	182.85	600.76	0.0001
D^2	119.04	1	119.04	391.1	0.0003
E^2	28.45	1	28.45	93.48	0.0023
F^2	1876.94	1	1876.94	6166.7	>0.0001
G^2	380.22	1	380.22	1249.22	>0.0001
ABC	0	0			
ABD	292.7	1	292.7	961.67	>0.0001
ABE	0	0			
ABF	0	0			
ABG	0	0			
ACD	0	0			
ACE	1.39	1	1.39	4.55	0.1225

(*Continued*)

Table 9 (***Continued***)

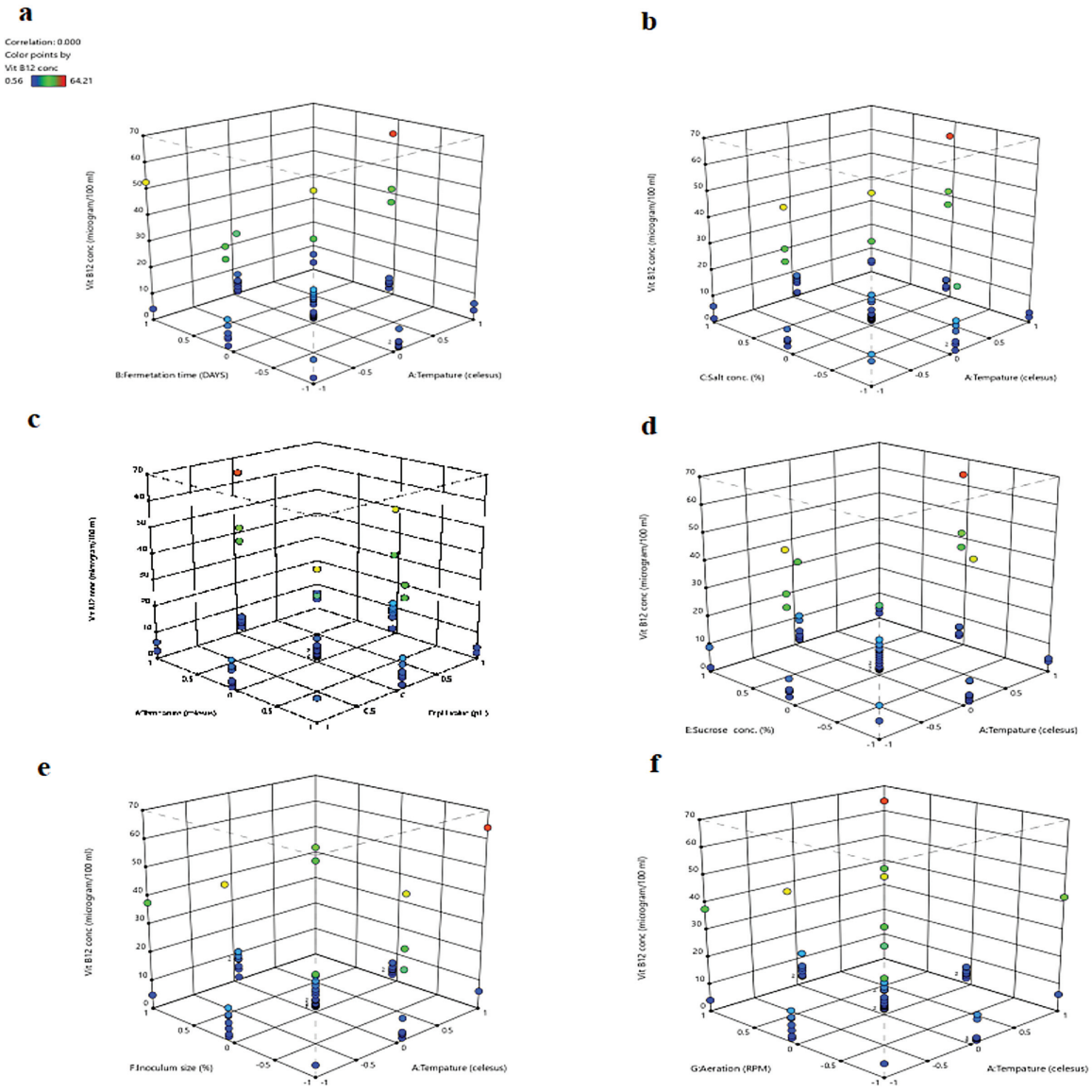
Source	Sum of squares	df	Mean square	F-value	P-value
ACF	0	0			
ACG	0	0			
ADE	0	0			
ADF	0	0			
ADG	0	0			
AEF	0	0			
AEG	0	0			
AFG	1990.8	1	1990.8	6540.81	>0.0001
BCD	0	0			
BCE	0	0			
BCF	65.44	1	65.44	214.99	0.0007
BCG	0	0			
BDE	0	0			
BDF	0	0			
BDG	0	0			
BEF	0	0			
BEG	0.3403	1	0.3403	1.12	0.3679
BFG	0	0			
CDE	0	0			
CDF	0	0			
CDG	5.54	1	5.54	18.22	0.0236
CEF	0	0			
CEG	0	0			
CFG	0	0			
DEF	35.49	1	35.49	116.6	0.0017
DEG	0	0			
DFG	0	0			
EFG	0	0			

Figure 8



The difference between actual and predicted values. The color scale represents the concentration of vitamin B_{12}.

Figure 9



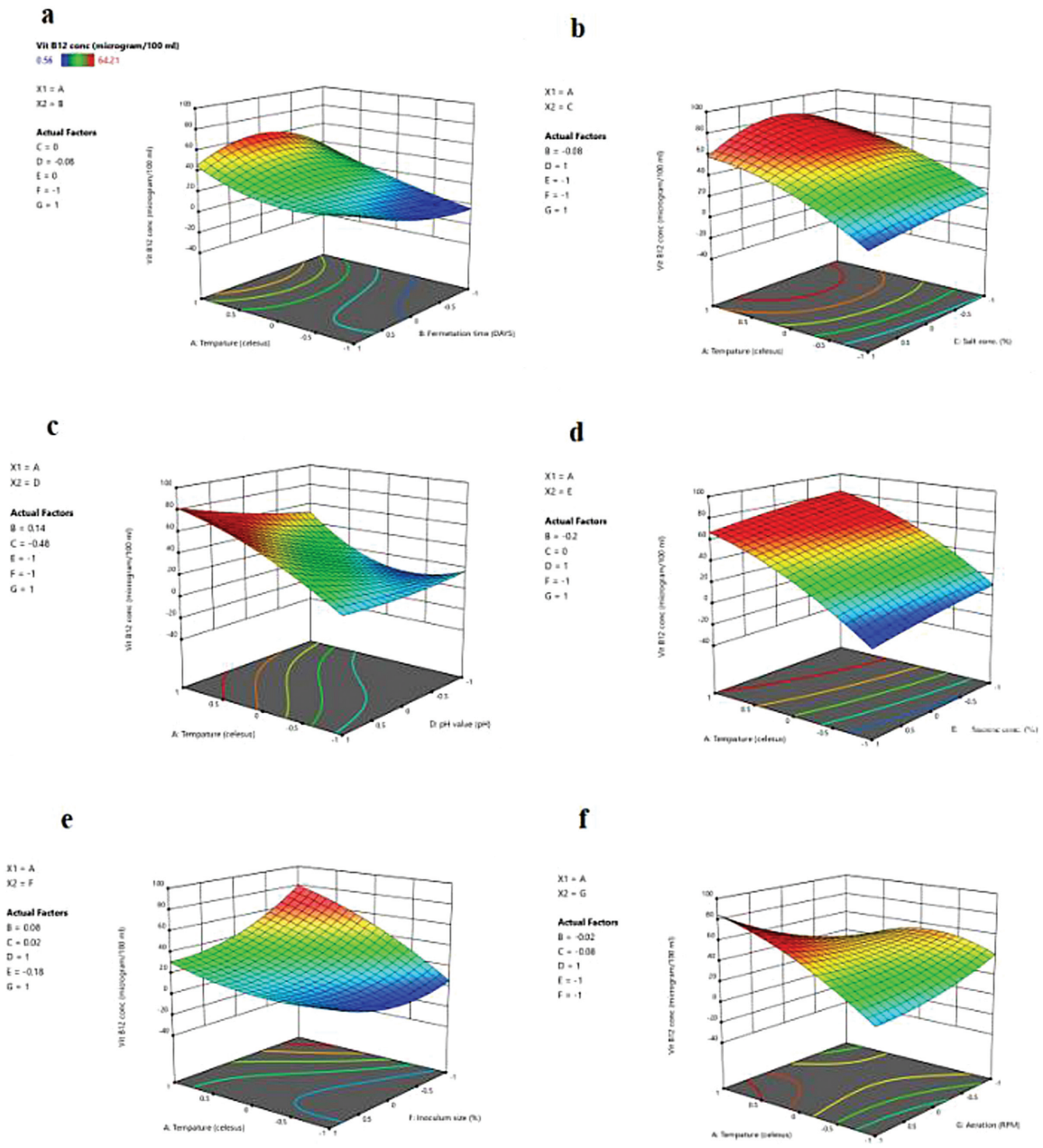
The response of the interaction between temperature and other parameters on the vitamin B_{12} yield. a. The effect of the interaction between temperature and fermentation time. b. The effect of the interaction between temperature and salt concentration. c. The effect of the interaction between temperature and pH value. d. The effect of the interaction between temperature and sucrose concentration. e. The effect of the interaction between temperature and inoculum size. f. The effect of the interaction between temperature and aeration. The color scale represents the concentration of vitamin B_{12}. The red point represents the highest yield of vitamin B_{12}.

Therefore, it is noticeable that increasing molasses concentration from 6 to 12% and adjusting the pH at 6.5 enhanced the productivity of vitamin B_{12} (Fig. 11).

Contrary to the obtained results in the current study (Table 7), applying molasses as production medium for vitamin B_{12} production reduced the strain productivity (14.31 to 3.49 µg/100 ml). This is in contrast with the productivity levels obtained when applying a production media containing sucrose as a carbon source (50.06, 53.07, and 64.21 µg/100 ml at run 16, 28 and 38, respectively) (Fig. 11). This was probably due to the presence of hydroxymethyl furfural and some inhibitors in molasses leading to the reduction of the vitamin B_{12} yield [46].

There is a necessary need for the optimization of the microbial fermentation process conditions to reach an optimal cost-effective medium, therefore Li *et al.*, 2013 used beet molasses to produce vitamin B_{12} by *Pseudomonas denitrificans* and yielded (181.75 mg/l of vitamin B_{12} in a 120 000 L fermenter). Additionally, Li *et al.*, 2013 reported that the production of vitamin B_{12} by molasses could not only has beneficial nutritional for the cell growth, it also reduced the cost of fermentation in a 120 000 L fermenter [62].

Figure 10



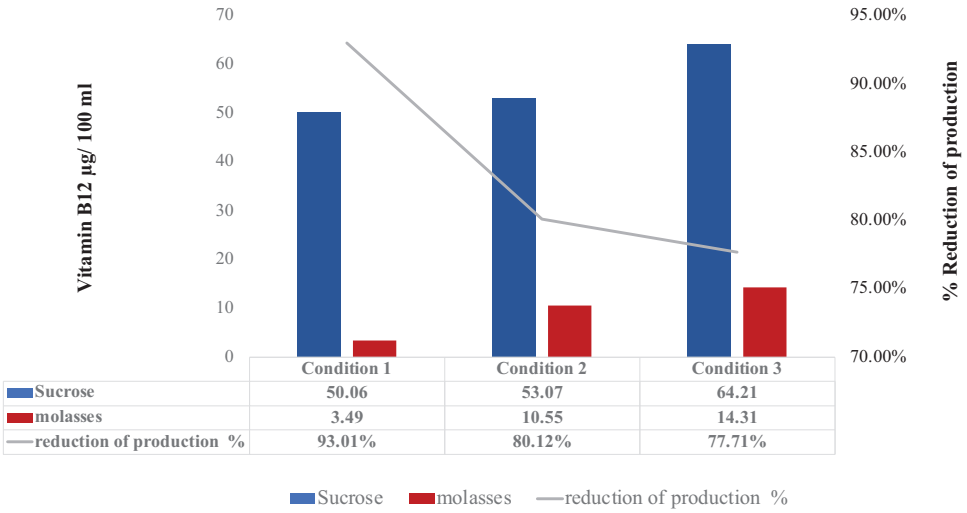
The three dimensional response surface and contour plots of the two-factor interaction effects on the yield of vitamin B_{12} production. a. The interaction between temperature and fermentation time. b. The interaction between temperature and salt concentration. c. The interaction between temperature and pH value. d. The interaction between temperature and sucrose concentration. e. The interaction between temperature and inoculum size. f. The interaction between temperature and aeration. The color scale represents the concentration of vitamin B_{12}.

Conclusion

Four yeast strains including *Candida pelliculosa* strain BYI and *Geotrichum candidum* strains MZYC, MZYD, and MZYG were selected for optimization of vitamin B_{12} production. *Geotrichum candidum* MZYD strain was selected for the statistical modelling using RSM to optimize seven factors for the vitamin B_{12} production. A maximum yield of 64.21 µg/100 ml was obtained using the optimized RSM conditions. These optimized conditions were used to produce vitamin B_{12} using molasses as a raw material for the microbial growth with a decreasing of maximum yield to (10.55 µg/100 ml of vitamin B_{12}). Therefore, yeasts could produce high yield of vitamin B_{12}, and molasses could be used as a production medium for vitamin B_{12} via yeast with further studies for optimizing and improving the yield.

Financial support and sponsorship
Nil.

Figure 11



Vitamin B_{12} production using molasses as a production medium compared with using artificial production media containing sucrose as a carbon source under three different conditions. The percentage of vitamin B_{12} productivity reduction when using molasses as a production medium was calculated. Condition 1 sucrose : 30°C, 2 days, 0% salt, pH 7.5, 6% sucrose, 10% inoculum, 110 rpm. Condition 1 molasses: 35°C, 2 days, 0% salt, pH 6.5, 12% molasses, 10% inoculum, 200 rpm. Condition 2 sucrose: 25°C, 4 days, 0% salt, pH 5.5, 12% sucrose, 5% inoculum, 110 rpm. Condition 2 molasses: 35°C, 2 days, 0% salt, pH 7.5, 12% molasses, 10% inoculum, 200 rpm. Condition 3 sucrose: 35°C, 2 days, 0% salt, pH 6.5, 12% sucrose, 10% inoculum, 200 rpm. Condition 3 molasses: 35°C, 2 days, 0% salt, pH 6.5, 12% molasses, 10% inoculum, 200 rpm.

Table 10 Optimization of the production medium with molasses

Strain MZYD conditions	Biomass g/100 ml.	Vitamin B12 µg/100 ml culture	Vitamin B12 µg g-1cells culture
12% molasses and pH 7.5	1.4	10.55***	7.54
12% molasses and pH 6.5	1.23	14.31***	16.675
6% molasses and pH 7.5	1.73	5.57***	3.22
6% molasses and pH 6.5	1.33	3.49	2.62

Each value represents a mean of three replicates. ****P* less than 0.001. Determination conditions: 12% sucrose as a carbon source, 10% inoculum size, 35C, 2 days fermentation, 0% salt, and 200 rpm.

Conflicts of interest

The authors declare there are no conflicts of interest.

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