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Developing and Optimizing Media Formulations Using Agro waste for the Production of Fungal Secondary Metabolites, Specifically Vitamin B12

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Abstract: *Vitamin B12 is a water-soluble compound that is essential for human health, typically sourced from animal products or synthesized through microbial fermentation. This study explored the formulation of mycological media from agro waste for production of Vitamin B12. The media formulated were agro waste from sugarcane and sweet potato (SSP) and sugarcane and cassava (SC). Experimental condition variables including different percentages (1% and 2%) of carbon ($Na_3C_6H_5O_7$) and nitrogen ($NaNO_3$) source, hydrogen ion concentration (pH:5,6,7and 8), different ratios of 5-6 dimethylbenzimidazole (5,6-DMB) and cobalt (Co) and temperature (30°C and 37°C) were optimized using standard procedures in order to enhance microbial production of vitamin B12. The fungi Aspergillus niger (A. niger) strains screened for production of vitamin B12 were obtained from soil sample collected from hospital dumping sites. Statistical analysis of data was done using ANOVA and results were presented as means ± standard deviations. Results obtained from optimization showed that temperature, pH, and different percentages of carbon and nitrogen have significant ($p \leq 0.05$) effects on fungal growth as well as its production of secondary metabolite (vitamin B12). The result also showed that the effect of different ratios of DMB and Co on vitamin B12 production was significant ($P<0.05$). The optimum conditions for the growth of A. niger and synthesis of its secondary metabolite were observed at temperature 30°C (0.4µg/ml±0.006), pH 7.0 (0.92µg/ml±0.007) DMB and Co ratio 80: 0.75 mg (0.94µg/ml± 0.006) in SC medium. The outcome of this study has indicated that vitamin B12 would be effectively produced by A.niger strains using formulations of agro waste: Sugarcane and cassava media supplemented with appropriate percentage of carbon and nitrogen, adequate ratio of DMB and Co and incubation at optimum temperature and pH values. These findings have significant implications for the food, pharmaceutical and biotechnology industries.*

Keywords: Agro waste, vitamin B12, optimization, *Aspergillus niger*, 5-6 dimethylbenzimidazole, Cobalt.

INTRODUCTION

Cobalamin's (Cbl) importance in human physiology and its widespread use in pharmaceuticals and food products have sparked intense global interest in understanding its origin (Hajfarajallah *et al*., 2014).Cyanocobalamin, commonly referred to as vitamin B12, is a water soluble compound that belongs to the Cbl family, distinguished by its large molecular size, with a weight of over 1000, making it the largest of the B complex vitamins. Methylcobalamine and 5-deoxyadenosyl-cobalamines are its two active forms (Ladesma-Amaro *et al*., 2013; Smith and Johnson, 2020). It consists of a corrinoid ring and a ligand both upper and lower (Fang *et al*., 2017). Its structure and biosynthesis is complex with over 30 phases of transformation (Calvillo*et al*., 2022). Cbl is a co-factor needed for fatty biochemical process, DNA synthesis, protein metabolism as well as initiation of hemoglobin (Kbyeh *et al*., 2019). Some bacteria and archaea possess this biosynthetic pathway, although the phyla capable of synthesizing vitamin B12 are not necessarily interrelated (Calvillo *et al*., 2022). Bacteria and fungi produce vitamin B12 and constitute the only source of the vitamin.

The recognition of pernicious anemia, a condition resulting from a lack of vitamin B12, which presents with symptoms like fatigue, memory loss, and headache, and which can progress to dementia and nerve damage in severe cases, was first identified in 1824, laying the groundwork for Minot and Murphy's groundbreaking discovery of vitamin B12 in the 1920s (Calvillo *et al*., 2022).Alhough, according to Calvillo *et al*. (2022) pernicious anemia patient could be treated with a nutritious diet found naturally in animal products.

The cost of producing vitamin B12 is high because it requires specialized culture media and a complex extraction process, which is made more difficult by the fact that the vitamin is present in very small quantities in the broth culture, making its extraction and purification a costly endeavor (Hajfarajallah *et al*., 2014; Kosmider *et al*., 2012). The use of low-cost and environmental friendly raw materials such as agro waste, industrial or food wastes could be ways to produce cost effective media for the synthesis of vitamin B12 (Hajfarajallah *et al*., 2014). Efforts have been made to improve the yield of vitamin B12 production through optimization routes. Vitamin B12 are produced from *Propionibacteria* from a variety of carbon source (Hajfarajallah *et al*., 2014), such as sucrose (Li *et al*,. 2008a, b), tomato pomace (Haddadian *et al*., 2001). Appropriate contents of nutrients are required in the broth medium for the biosynthesis of the vitamin.

Members of the fungi family are capable of producing a wide variety of natural products, but the extent to which these products benefit the fungi themselves is still a topic of investigation and remains unclear (Okpalauwaekwe *et al.*, 2020; Calvo*et al*., 2002). However, interest in some of these natural products is considerable, as many secondary metabolites are of medical, industrial and agricultural importance (Calvo *et al.*, 2002). According to Fabrizio *et al*., (2017), all the natural products synthesized by fungi, special interest is given to vitamin B12, antibiotics and lovastatin due to the reduction in the potency of existing drugs used to treat anemia, cholesterol and cardiovascular diseases, which poses a major threat to health security globally.

Research has shown that agricultural waste can be effectively used as a substrate for fungal culture, leading to the production of various valuable natural products. Examples of this include the production of cellulose by certain fungi grown on pineapple waste (Omojasola *et al.,* 2008), carotenoids produced by *Blakeslea trispora* cultivated on agricultural waste (Papaioannou *et al.*, 2012), enzyme production by *Aspergillus niger* on agricultural waste (Milala *et al.*, 2005), energy source for production of lipase by *Aspergillus fumigatus* on agricultural waste (Naqvi *et al.,* 2013) and a range of other valuable natural products produced by filamentous fungi using agricultural wastes as a substrate (Arushdeep and Umar, 2014).

Fungi are heterotrophic in nature and as such, requires carbon, nitrogen and energy source for metabolism and survival. Basic nutritional needs of fungi and yeast could be met when supplied with an aerobic environment, glucose, ammonium salt, inorganic ion growth factors to enhance their survival (Smith, 2014). Nutritional requirements are necessary for cultivation and optimization of fungal growth for increased metabolites production.

The agricultural waste may meet these requirements and work as fungal growth medium for biosynthesis of secondary metabolites and can replace expensive, scarce, carcinogenic and mutagenic media in markets (Umedum and Anaejekwute, 2017). A lot of literature aboundson the production of vitamin B12 using bacteria, but few has been reported on fungi. The present study aimed at developing and optimizing media formulation using agro waste for the production of fungi secondary metabolites (vitamin B12).

MATERIALS AND METHODS

Collection of samples: A total of 50 soil samples from garden, hospital waste dumping site and abattoir were randomly collected from different sites in Mubi South L.G.A, Adamawa state. The soil samples were collected from the superficial layer of the soil at depth not exceeding 3-5 cm, where most of the fungal population is concentrated. Soil samples were collected (approximately 100 g) in clean, dry and sterile container using sterile spatula. The samples that were collected were transported to the laboratory within 1 hr of their collections for mycological analysis.

Processing of sample: One gram of each sample was added into 10 ml sterile test tubes, 3 ml of normal saline was added into the sample and mixed thoroughly by manual shaking. Then the volume of the resulting solution was made up to 10 ml using normal saline. Tenfold serial dilution was carried out using normal saline as diluents. One milliliter solution from 10^{-1} dilution was transferred into another test tube containing 9ml of normal saline to get 10^{-2} dilution and other test tubes repeatedly to get 10^{-4} dilution.

Isolation of fungal organisms: One tenth milliliter (0.1ml) inoculums each was aseptically collected from 1:1000 dilution and plated on Sabourand Dextrose Agar (SDA) containing 0.05% chloramphenicol, using spread plating method. The inoculated plates were incubated inverted at ambient temperature (30 ± 2^0C) for 3-5 days after which the colonies from the culture were sub cultured on Sabourand Dextrose Agar (SDA) and Potato Dextrose Agar (PDA) supplemented with 0.05% chloramphenicol to obtain pure chemicals (Okpalauwaekwe *et al*., 2020).

Identification of Fungal Isolates: The fungi that were isolated were purified, characterized and identified based on the macroscopic, microscopic and molecular characteristics (Watanabe, 2010). Result not shown.

Macroscopy: The colonies that were obtained were carefully examined for fungal characteristics such as colour, texture, consistency of the growth and other peculiar features of the colonies (Watanabe, 2010). Result not shown.
Microscopy: This was carried out using needle mount technique. A drop of lacto phenol cotton blue (LCB) solution was placed on the centre of a clean grease-free slide. A fragment of the colony was mixed with the LCB using sterile wire loop and then covered with cover slip. Air bubble was avoided and excess fluid from the outside of the cover slip was wiped with cotton wool and the slide was passed through the flame to warm the staining so as to remove the remaining air bubbles and facilitate staining of the fungal element. The slide was thenexamined under the microscope using x10 objective lens and then viewed with x40 objective lens, which revealed the nature of the hyphae, shape, size, texture and conidial arrangement. The pictorial nature of the fungal organisms was confirmed using fungal atlas (Watanabe, 2010). Result not shown

Molecular characterization: This involved extraction and purification of DNA from the isolates. Then the quality of the DNA was determined using mass spectrophotometer. This was followed by amplification of the DNA using Polymerase Chain Reaction (PCR) machine. The amplicons that were obtained were sequenced using ABI sequencer and chromatograms that were obtained were cleaned and BLAST over the internet (Ogbuji *et al.*, 2021). Result not shown.

Screening of the Fungal Isolates for Production of Vitamin B12
The fungal isolates were grown in potato dextrose broth (PDB) containing 5.0g peptone, 10.0g glucose, 0.01g cobalt chloride and 1 liter of water adjusted at pH= 7.0.
One hundred milliliters of the medium was dispensed into 250ml cotton plugged Erlenmeyer flasks. Each flask was incubated with 1.0×10^6 cell/ml of the fungal isolates prepared according to Mc Farland standard and incubated at 30°C for 4 days as static culture then 4 days as submerged culture.
Then, 5-6 Dimethyl benzimidazole (DMB) was added as B12 precursor, to the fermentation cultures 24h before the end of incubation period. After incubation, the cultures were centrifuged using ultra high centrifuge at 8000 rpm and tested for the presence of vitamin B12 using ultra violet visible spectrophotometric method at 240nm (Abou- Tales Khadiga *et al.*, 2012).

Collection, Preparation and Formulation of Medium

Sugar cane, sweet potato and cassava peels were collected from Mubi south L.G.A. The peels were collected in a clean polythene bags and were taken to the laboratory. The peels were washed and air-dried and pulverized into powdered form. Twenty grams comprising 10g each of the sugarcane and sweet potato (SSP) as well as cassava and sugarcane (SC) samples were weighed

into 400ml of distilled water in 1000ml Erlenmeyer flask and allowed for 7 days, after which the mixture was filtered. Then 100 ml of the filtrate was used.

Optimization of Carbon source, Nitrogen source, pH and Temperature for Production of Vitamin B12.

The effect of 1%, 2% of carbon sources and nitrogen source were investigated by supplementing the agro waste media with the carbon and nitrogen sources at different pH. The pH (5, 6, 7, 8) and temperature (30°C, 37°C) for vitamin B12 production (Hooi and Lee, 2014; Suganthi*et al.*, 2014).

Statistical Analysis: The data generated from this study were presented in Tables as means ± standard deviations. The statistical significance of the data generated were ascertained using one-way Analysis of variance (ANOVA) whereas the means that differed significantly were identified using Least Significance Difference (LSD) and Duncan tests of significance.

RESULTS

	Concentration (µg/ml)	
pH	SSP	SC
5.0	0.34 ± 0.006^{a}	0.53 ± 0.007^{a}
6.0	0.39 ± 0.005^{b}	0.57 ± 0.008^{b}
7.0	0.68 ± 0.023^{c}	0.92 ± 0.007^{c}
8.0	0.45 ± 0.042^{d}	0.59 ± 0.006^{d}

Process optimization of vitamin B12 production by *Aspergillus niger* (*A. niger*) in agro waste media supplemented with 1% Carbon ($Na_3C_6H_5O_7$,) and Nitrogen ($NaNO_3$), 80mg 5,6-Dimethyibenzimidazole (DMB) and 0.75mg Cobalt (Co) at different pH.

Table 1: Effect of pH on vitamin B12 Production

Key:

SSP = Sugar and Sweet potatoes media

SC = Sugar cane and cassava media

Mean values in the same column having different letters of alphabet as superscripts are significantly different at the 5% level of confidence ($P \leq 0.05$).

	Concentration(µg/ml)	
pH	SSP	SC
5.0	0.29±0.006[a]	0.40±0.006[a]
6.0	0.32±0.006[b]	0.46±0.062[a]
7.0	0.56±0.006[c]	0.72±0.007[c]
8.0	0.39±0.006[d]	0.31±0.005[d]

Process optimization of vitamin B12 production by *A. niger* in agro waste media supplemented with 2% Carbon ($Na_3C_6H_5O_7$,) and Nitrogen ($NaNO_3$), 80 mg 5,6- DMB and 0.75 mg Co at different pH.

Table 2: Effect of pH on vitamin B12 Production.

Key:

SSP = Sugar and Sweet potatoes media

SC = Sugar cane and cassava media

Mean values in the same column having different letters of alphabet as superscripts are significantly different at the 5% level of confidence (P≤0.05).

	Concentration(µg/ml)	
Ratio: DMB/Co (mg)	SSP	SC
50/0.25	0.16±0.004[a]	0.34±0.011[a]
65/0.50	0.44±0.006[b]	0.50±0.006[b]
80/0.75	0.68±0.007[c]	0.94±0.006[c]
95/1.00	0.55±0.006[d]	0.74±0.036[d]

Process optimization of vitamin B12 production by *A.niger* in agro waste media supplemented with different ratios of DMB and Co

Table 3: Effects of different ratios of DMB and Co on vitamin B12 production

Key:
SSP = Sugar and Sweet potatoes media
SC = Sugar cane and cassava media
Mean values in the same column having different letters of alphabet as superscripts are significantly different at the 5% confident level (P≤0.05)

	Concentration(µg/ml)	
Temperature	SSP	SC
30°C	0.68±0.007[a]	0.94±0.006[a]
37°C	0.60±0.009[b]	0.89±0.0012[b]

Process production of Vitamin B12 by *A. niger* in agro waste media supplemented with 1% Carbon ($Na_3C_6H_5O_7$,) and Nitrogen ($NaNO_3$) 80 mg DMB and 0.75mg Co at pH 7 and at different temperature.

Table 4: Effects of different temperatures on Vitamin B12 production by *A. niger*

Mean values in the same column having different letters of alphabet as superscripts are significantly different at the 5% level of confidence ($P \leq 0.05$).

Discussions

From the findings of the research it was found that isolates from hospital dumping sites (General hospital and new life clinic Mubi) which were strain of filamentous fungi *Aspergillus niger (A.niger)* which have been previously characterized produced a significant yield of the vitamin B12 on the conventional media during screening as well as on the agro waste media though the yield was low. This is in line with the report of Abou- Taleb *et al* .(2012) that the low yield of vitamin B12 could be due to nutrient insufficiency, but when supplemented with 1% sodium nitrate ($NaNO_3$) and sodium citrate ($Na_3C_6H_5O_7$), the concentration of vitamin B12 increased. The finding also supports the work of Okpalauwaekwe *et al*. (2020); and Smith, (2014) that for maximum production of valuable bioactive compounds, providing the right nutrients in the right amount is essential for fungal growth. By optimizing nutritional requirements, fungal growth was enhanced and the production of these valuable compounds was boosted. The result obtained also supports the concept that *A.niger* harbors a vast array of highly productive genes that are effective in the biosynthesis of a wide range of valuable natural products, which are useful in medicine, food, and agriculture, making it a valuable resource for various industries (Yu *et al*., 2021). The production of vitamin B12 by A. niger using the agro waste media from sugarcane and cassava peels (SC) yielded a significant concentration of vitamin B12 than that produced from sugarcane and sweet potatoes (Tables 1, 2 and 3). This finding supported the work of Olutosin and Kayode (2021); Obadina *et al.* (2006), that cassava peel is a remarkable substrate that can be utilized for *A. niger* cultivation and alternative source of vitamins production.The use of *A. niger* in the food and pharmaceutical industrial production have been assessed as acceptable for daily intake by the World Health Organization (Olutosin and Kayode, 2021; Cordenunsi*et al.*, 2014). The United States Food and Drug administration under the Federal food, drug and cosmetic act also recognized *A.niger* fermentation as safe (Olutosin and Kayode, 2021; Howard and Denton, 2010).The growth

and nutritional condition of the media were optimized to enhance the production of vitamin B12. The agrowaste media formulated were SSP and SC media and were optimized based on pH, temperature, percentages of carbon and nitrogen source as well as the ratios of Co and DMB. The concentration of vitamin B12 on SSP and SC supplemented with carbon and nitrogen sources revealed that 1% $Na_3C_6H_5O_7$ and 1% $NaNO_3$ produced significant ($P \leq 0.05$) concentration of vitamin B12 on SC at (0.92±0.007 µg/ml) and SSP (0.68±0.02 ug/ml) than at 2% SC (0.72±0.007µg/ml) and SSP (0.56±0.006 µg/ml). The finding is consistent with the research conducted by Okpalauwaekwe *et al.*(2020); smith (2014) that nutritional requirements are necessary for cultivation and optimization of fungal growth for increased bioactive compound production. Also the concentration of vitamin B12 was high when supplemented with 1% carbon and nitrogen source, 80mg of 5-6 dimethylbenzimidazole (DMB) and 0.75 mg of cobalt (Co) on agro waste medium SC (0.94±0.006 µg/ml) than SSP (0.68±0.007 µg/ml) respectively. Our results match what Zhang *et al,* found in their 2023 study, these researchers supplemented different ratios of 5,6-DMB and Co on ruminal animal feeds.

The high concentration of vitamin B12 was obvious when the agro wastes were supplemented with 1% $Na_3C_6H_5O_7$ and $NaNO_3$, 80mg of DMB, 0.75 mg of Co at pH7 and at temperature of 30^{o}C.This is in line with the reports of Joshi *et al*. (2017) that fungi grow best at an optimum temperature range of 25^{o}C- 30^{o}C.Besides, the best result of vitamin B12 production was recorded from the agro waste medium SC which is in consistent with the report of Olutosin and Kayode, (2021);Obadina *et al.*(2006). Much has been exploited on the production of vitamin B12 using bacteria, but less has been done on fungi. From this research findings, vitamin B12 which is typically produced industrially through fermentation of *Pseudomonas denitrificans* and *Propionibacterium freudenreichii,* could also be produced using strain of *A. niger* and agro waste media offering a promising alternative method to boost production, as this essential vitamin can only be naturally synthesized by microorganisms during fermentation, not through chemical synthesis (young *et al.*, 2021). This will meet up with response to commercial demand, environmental concern and food security.

The utilization of agro waste in production is on the increase as most agro waste contain phytochemical compounds, sugars and minerals that serve as raw material for some industrial production rather than waste (Olutosin and Kayode, 2021).

CONCLUSION

This research demonstrates the potential of utilizing agro waste as sustainable substrate for the production of vitamin B12 through microbial fermentation. The optimization of media formulation using sugarcane and sweet potatoes as well as sugarcane and cassava resulted in a significant increase in vitamin B12 yield. The optimized media composition and fermentation conditions can be scaled up for industrial production, providing a cost- effective and eco- friendly alternative to vitamin B12 production methods.

Recommendations:
Further research should be carried out to explore potential of producing other valuable compounds alongside vitamin B12 as well as to also improve strain of *Aspergillus niger* by genetic engineering to increase vitamin B12 yields.

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