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OPTIMAL CONDITIONS FOR VANILLIC ACID PRODUCTION BY LOCAL ISOLATE *ASPERGILLUS TERREUS* MD10–3(0)

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Abstract

A local fungal isolate *Aspergillus terreus* MD10-3(0) was optimized for vanillic acid production which was (14.9) mg/ml. by using 20 g/L glucose, (2.5) g/L ammonium sulfate, with (1.5) g/L pomegranate peels powder as a substrate, pH (5), (30) C, (1×10^6) cfu/ml, (150) rpm, and (7) days of incubation.

***Keywords*:** *Aspergillus terreus*, vanillic acid production, optimized conditions.

Introduction

Vanillic acid is a phenolic, aromatic compound. It is an organic, chlorogenic acid that occurs naturally. It is an oxidized form of vanillin so it is considered as a flavoring agent which is used widely in food industry. Vanillic acid can be used as a preserving food additive due to its antioxidant activity. Moreover, it is involved in medical and pharmaceutical industries due to its features being cardio protective and antibiotic (Almeida *et al*., 2016; Clavijo *et al*., 2008). According to (www. Pubchem.com), vanillic acid is 4-Hydroxy -3-Methoxybenzoic acid. Its molecular formula is $C_8H_8O_4$ with a molecular weight that equals (168.148 g mole). Vanillic acid can be produced by microbial or enzymatic conversion of some natural substrates like ferulic acid, eugenol or isoeugenol. Therefore, it has great industrial applications in cosmetics, food industry, beverages, animal feed and pharmaceutical industry. (Noa and Mehlana, 2018; Ashengroph and Esfahani, 2008). Ashengroph and Esfhani (2008) also mentioned that isoeugenol is a cheap alternative which can be used instead of ferulic acid, as natural substrates, to produce vanillin. Yet, its productivity is relatively low due to the toxicity of the substrate and its oxidation from vanillin to vanillic acid. As such, it can be considered a good, cheap source to produce vanillic acid. However, these processes cannot be done without exploiting the bioconversion activities of some fungi, yeast and bacteria.

Lesage-Meessen *et al*. (1998) added that biological processes that give aromatic compound make use of some microorganisms, animals, plant cells or their enzymatic systems when they involve one of the degradation outputs of substrates that contain aromatic ring. They believe that vanillic acid and even vanillin can be produced by bioconversion processes from ferulic acid in a medium which contains phospholipids. Yet, the medium should be cultured with at least one of the classes of the fungi: Ascomycetes, Basidioymycetes or Actinomycetes.

Moreover, to increase the yield of bioconversion of ferulic acid, we can activate peroxysomal beta-oxidation. As for the usage of vanillic acid in food industry and pharmaceutical industry to improve flavor, vanillic acid is employed as an additive by co-crystallizing the amines with it due to its sweet flavor, and pleasant smell. However, vanillic acid is soluble in alcohols, ethers and amines when they are used as solvents. Vanillic acid has a great tendency to form new crystalline materials with amine molecules due to its high hydrogen bond which is located in its COOH group site. (Noa and Mehlana, 2018)

Materials and Methods

Optimal conditions of VA production

A number of parameters are considered to determine the best production of VA from the chosen fungal isolate. Basal media used contained: 20 g/L maltose; 1.842 g/L di-ammonium tartrate; 0.5 g/L yeast extract; 0.2 g/L KH_2PO_4; 0.2 g/L CaCls; 0.0132 g/L of $2H_2O$; 0.5 g/L $MgSO_4$. pH was adjusted on 5.6 (Tan *et al*., 2015). The mixture was autoclaved on (121 C) for 15 minutes. Some detectors like the quantity of the resulted VA, dry biomass weight, and pH were determined for each parameter after finishing incubation period.

Carbon source and its concentration

Basal media mentioned previously was prepared by using different carbon sources and its concentrations:

a- Chosen Carbon sources involved: maltose, glucose, date syrup and molasses. Each of them was added separately (three different concentrations for each carbon source; 1%, 2%, 3%). Incubation was done in the shaker incubator on 150 rpm, and 30 C. In the fourth day of incubation period, three types of substrates were added separately for each concentration of each carbon source: (Ferulic acid, dry peels of pomegranate, and dry peels of grapefruit of 0.5 g/L). Dry biomass weight was calculated. Also, pH was measured for the basal media of production and VA was determined quantitatively. As such, the best carbon source and the best substrate for production was determined and chosen.

b- After determining the best carbon source and substrate, different concentrations of the substrate chosen previously for the best production were tested in the same conditions. Different concentrations of the chosen carbon source were studied by using 1%, 2%, and 3% concentrations and the chosen substrate was 0.5 g/L. Different concentrations of the chosen substrate were determined by using 0.5, 1, 1.5, and 2 g/L.

Nitrogen source and its concentration

Basal media was prepared and the best previously determined and chosen carbon source was added with its best productive concentration to the basal media. Also, the best substrate was added with its best concentration. As such, the best nitrogen source was determined as follows:

a- In addition to the di-ammonium tartrate which was the nitrogen source used in the basal media, another nitrogen sources were added separately but with equal concentrations. They are (Peptone, ammonium sulfate, sodium nitrate, yeast extract). The concentration for each source was 1.842 g/L as recommended by Tan et al., (2015). The primary pH for the media was adjusted on (5.6). These sterilized and inoculated media with fungal isolate at the age of 7 days, 1% concentration and (1×10^7) cfu/ml were incubated on (30 C) for 7 days. After incubation period, the basal media was filtered for each flask used and the dry biomass weight was determined. Also, pH was determined for the production media and VA production was determined quantitatively .

b- After determining the best nitrogen source, different concentrations of nitrogen source which was already determined for the best production were tested in the same conditions. The concentrations were (1 g/L, 1.842 g/L, 2.5 g/L, 3 g/L)

pH

Basal media was prepared again and the best previously determined and chosen carbon source and nitrogen source were added with their best previously tested concentrations. The pH of the flasks was adjusted on (4,5, 5, 5.6, 6, 6.5) by using 0.1 N of NaOH or HCl. The sterilized and inoculated flasks were incubated on (30 C) for 7 days. After incubation period, the basal media was filtered for each flask by sterilized filter unit supplied by filter paper. The dry biomass weight was determined and the pH was determined for the basal media of production. Also, VA was determined quantitatively.

Temperature of Incubation

The effect of incubation temperature was studied for the chosen isolate for producing VA by using the basal media. The temperature degrees chosen were (28, 30, 35, 40) C. The best carbon and nitrogen sources with their best concentrations were added to the basal media of production. The pH was adjusted on the best previously determined pH. The sterilized and inoculated flasks were incubated on the four chosen temperature degrees separately and respectively. After incubation period, the basal media was filtered for each flask by sterilized filter unit supplied by filter paper. The dry biomass weight was determined and the pH was determined for the basal media of production. Also, VA was determined quantitatively.

Size of Inoculation

Basal media was prepared and the best previously determined and chosen carbon source and nitrogen source were added with their best previously tested concentrations. The pH was adjusted on the best previously determined pH. The flasks which contain the sterilized media were inoculated with different numbers of spores as follows: (1×10^4, 1×10^5, 1×10^6, 1×10^7 cfu/ml). These flasks were incubated on the previously determined temperature for best production for 7 days. After incubation period, the basal media was filtered for each flask by sterilized filter unit supplied by filter paper. The dry biomass weight was determined and the pH was determined for the basal media of production. Also, VA was determined quantitatively.

Aeration

To determine the best aeration for producing VA from the chosen isolate in this study, the basal media was prepared. The best previously determined and chosen carbon source and nitrogen source were added with their best previously tested concentrations. The pH was adjusted on the best previously determined pH. The flasks which contain the sterilized basal media were inoculated with the best inoculation size as previously determined. They were incubated on the best temperature for producing VA but the shaker incubator was adjusted on different rpm(s) for each time as follows: (120, 150, 200 rpm) respectively in the minute. After incubation period, the basal media was filtered for each flask by sterilized filter unit supplied by a filter paper. The dry biomass weight was determined and the pH was determined for the basal media of production. Also, VA was determined quantitatively.

Period of Incubation

The basal media was prepared according to the previous results of treatments and inoculated at (6, 7, 9, 12) days. The fermented media was filtered for each flask by sterilized filter unit supplied by a filter paper. The dry biomass weight was determined and the pH was determined for the basal media of production. Also, VA was determined quantitatively.

Extraction of VA

Extraction of VA was performed according to the method described by Krygier *et al*., 1982. The extraction began with filtering the basal media by using a sterilized filter unit supplied with a filter paper (Millipore) 0.45. The pH was then adjusted on 2 and centrifuged (5000 xg). Then the residues were left and the supernatant was taken to be purified by hexane (1:1) using separation faunal.

After that, hexane extracts were left and the suspension was taken. Two solvents were mixed; diethyl ether; and ethyl acetate (1:1) and added to the suspension (1:1:1) gained in the last step. The mixture was put into a separation faunal for 14 minutes. The supernatant resulted was yielded and 2% of sodium sulfate was added to it. After filtration, rotary evaporator was used on (30 C).

Qualitative determination of VA production

High performance liquid chromatography analysis was done to *Aspergillus terreus* isolate production to assure the quality of VA. This technique was used in the determination of VA which was extracted from the selected isolate for the present study. (20 µl) of the sample was injected in the column type C18 (250× 4.6) mm, and (5) micron of particle size. The mobile phase used was methanol: water (50:50%) and the flow rate used was 1 ml/min. After that, absorbance was measured on the wave length 260 nm and the relationship between the peak and retention time was drawn. Retention time of the model sample was compared with the time of peak appearance of the standard sample. These

results were similar to what was found by Muti and Olimat (2018).

Results and Discussion

Optimized conditions of VA production

arbon source and its concentration:

Figure (1) shows the usage of different carbon sources with different concentrations to produce VA. It clarifies the excellence of glucose as the carbon source and pomegranate as a substrate on the other sources with the concentrations: 20 g/L and 0.5 g/L respectively. The production of VA reached (5.239) mg/ml. Figure (2) shows the dry biomass value resulted from the usage of glucose with 20 g/L concentration is high but the dry biomass of glucose 20 g/L with grape fruit peels powder gave higher number.

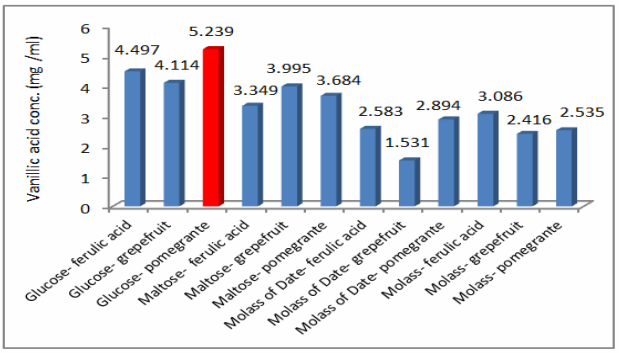


Figure (1) The effect of different carbon sources on VA Production by *Aspergillus terreus* MD10-3(0)

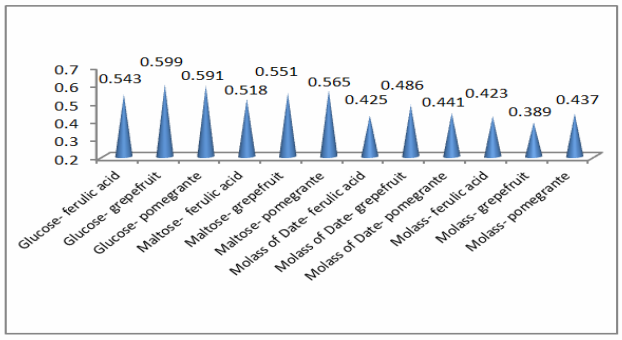


Figure (2) Dry biomass values of *A. terreus* MD10-3(0) by using different carbon sources

After determining the best productive carbon source, figure (3) shows different concentrations of glucose (1%, 2%, 3%) with 0.5 g/L of pomegranate. Glucose with 20 g/L was the best productive concentration. The dry biomass was calculated for the three concentrations and showed the excellence of glucose 20 g/L as well, as shown in the figure (4).

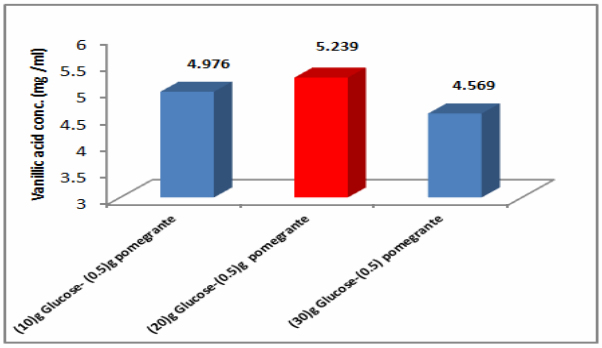


Figure (3) the effect of different concentration of carbon Sources on VA production by *Aspergillus terreus* MD10-3(0)

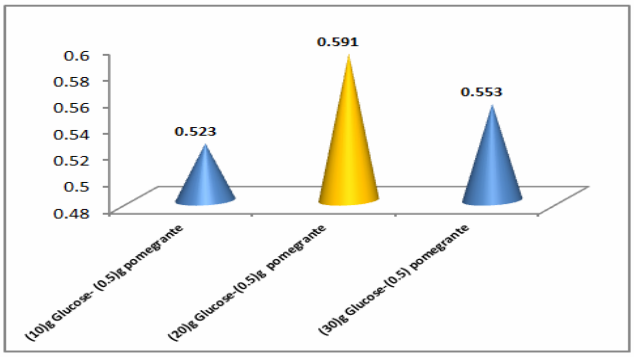


Figure (4) Dry biomass values of *A. terreus* MD10-3(0) by using different concentrations of carbon sources

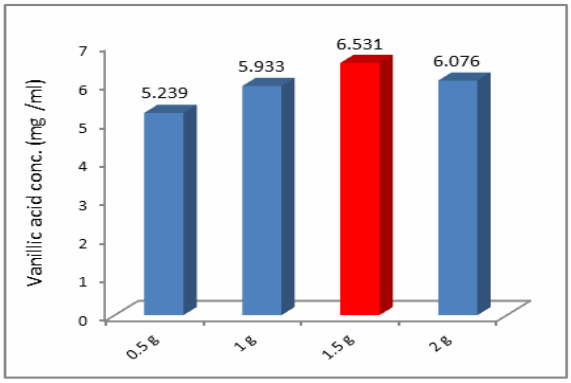


Figure (5) the effect of different concentrations of pomegranate On VA Production by *Aspergillus terreus* MD10-3(0)

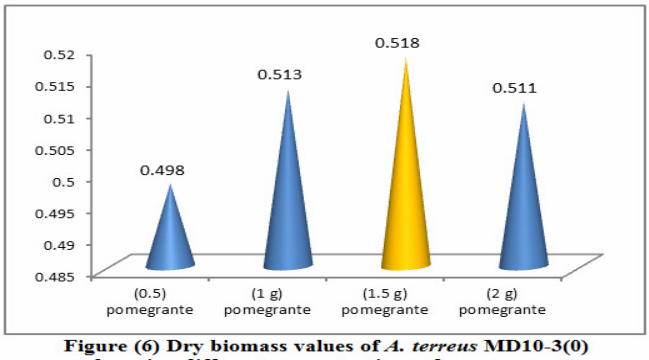


Figure (6) Dry biomass values of *A. terreus* MD10-3(0) by using different concentrations of pomegranate

The excellence of glucose on the other carbon sources could be explained for its high and fast metabolism. It motivates microorganisms to do its biological processes actively. Also, it worth mentioning that the usage of natural sources like pomegranate, grape fruit etc., peels powder as a substrate helps in decreasing the residues. (Al-Ani, 2005).

After choosing the glucose with the concentration 20g/L as the best carbon source for VA production, as the previous figures showed, four concentrations of the substrate pomegranate were tested as shown in Figure (5) the concentration 1.5 g/L gave the highest productivity of VA which was 6.531 mg/ml. Figure (6) presents the dry biomass achieved by using the four different concentrations of pomegranate which also gave the highest value at the same concentration (1.5 g/L). The mycelium value was 0.518 mg/100 ml.

By considering these results, glucose of 20 g/L with pomegranate 1.5 g/L are chosen being the best carbon source with the best concentration and the best substrate with the

best concentration to be recommended for further experiments in this study.

However, the results of the current study differ from what was given by Topakas *et al*. (2003) who mentioned that the usage of glucose to produce VA gave 4024 mg/L. this difference can be attributed to the type of the microorganism exploited in the bioconversion process. Tan *et al*. (2015) gave a near value of VA production from the current study which was 13 g/L but by using maltose as a carbon source.

Nitrogen source and its concentration

Figure (7) presents the usage of different nitrogen sources of 1.842 g/L concentration for each one to produce VA. Ammonium sulfate gave the highest productivity which reached 7.44 mg/ml. Figure (8) shows the value of the dry biomass for each nitrogen source and it was the highest for ammonium sulfate as well 0.497 mg/100 ml.

Furthermore, different concentrations of the best nitrogen source ammonium sulfate were tested as shown in figure (9). (2.5 g/L) gave the best productivity of VA which reached 8.63 mg/ml VA. Figure (10) displays the value of the dry biomass for using the different concentrations of ammonium sulfate. The dry biomass for the concentration 3 g/L of ammonium sulfate was the highest. This can be explained that productivity of VA is not proportional with the dry biomass.

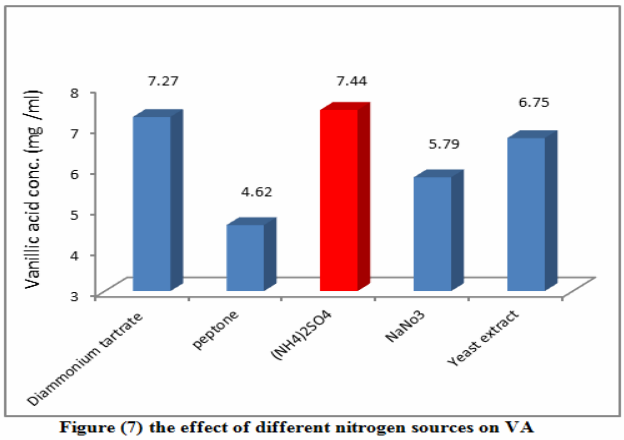


Figure (7) the effect of different nitrogen sources on VA Production by *Aspergillus terreus* MD10-3(0)

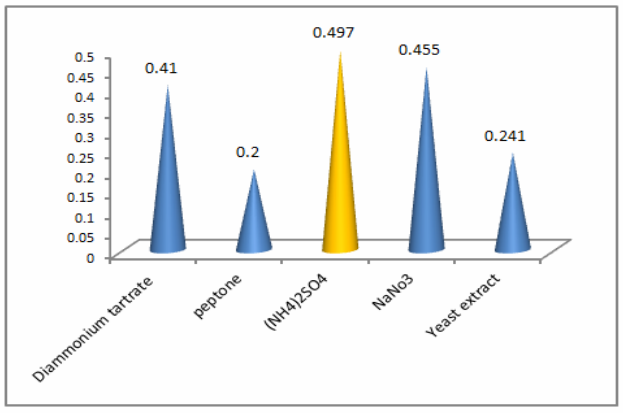


Figure (8) Dry biomass values of *A. terreus* MD10-3(0) By using different concentrations of nitrogn sources

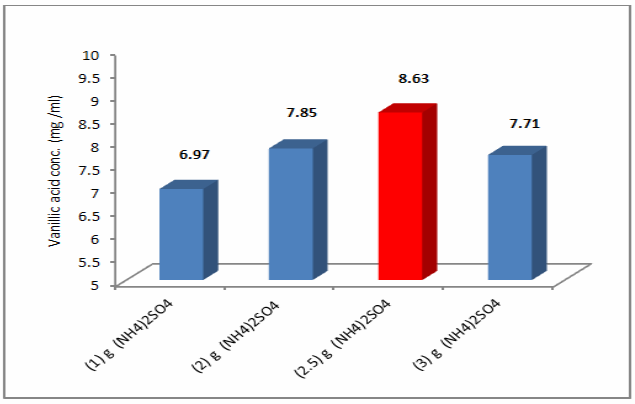


Figure (9) the effect of different concentrations of ammonium Sulfate on VA Production by *Aspergillus terreus* MD10-3(0)

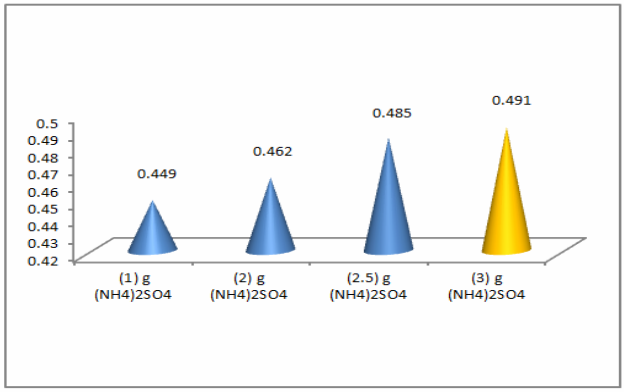


Figure (10) Dry biomass values of *A. terreus* MD10-3(0) by using different concentrations of ammonium sulfate

According to the present results, ammonium sulfate is chosen as the best nitrogen source for VA production with 2.5 g/L concentration. This can be recommended for further studies. Tan *et al*. (2015) and Topakas *et al*. (2003) have used different nitrogen sources for VA production. The first researchers used di-ammonium tartrate with 1.842 g/L concentration. The second researchers used yeast extract as the sole source of nitrogen. The type and concentration of the nitrogen source affect the productivity of VA depending on the type of microorganism. Ammonium sulfate could be faster to reach inside the cells of the microorganism than other nitrogen sources.

pH

pH effect on VA production from the fungal isolate *Aspergillus terreus* MD 10- 3(0) was studied. It is clearly noticed in the figure (11) that the best pH for VA production was 5. The production was 11.88 mg/ml. Yet, the dry biomass for *A. terreus* decreases when the pH goes higher than 5 as shown in the figure (12). Also, VA production decreases. This decrease in the dry biomass value for the fungi under study can be attributed to that this type of fungi grows well in acidic conditions with range from pH 3 to 6. However, the decrease in VA production can be attributed to the decreasing of the bioconversion enzyme activity which is responsible for VA production. Enzymes are proteins which contain ionized groups which affect their components and activity on the pH for the culture media (Lekha and Lonsane, 1997).

The researchers Lopez Malo (1998) and Argaiz (1997) in separated researches have studied the effect of pH on fungal growth and VA productivity. They assured that the

fungi grow on a wide range of pH ranging between (3-7). However, the fungal productivity depends on the pH which was determined by them to be (5- 5.5). This agrees with the results in the present study that pH 5 is the best for *A. terreus* growth and productivity. Also, Buswell *et al.* (1982) assured that pH 5.5 is the best for the growth of soft rot, and white rot fungi.

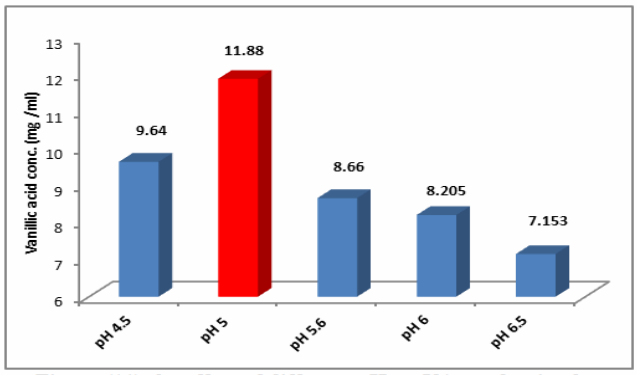


Figure (11) the effect of different pH on VA production by *Aspergillus terreus* MD10-3(0)

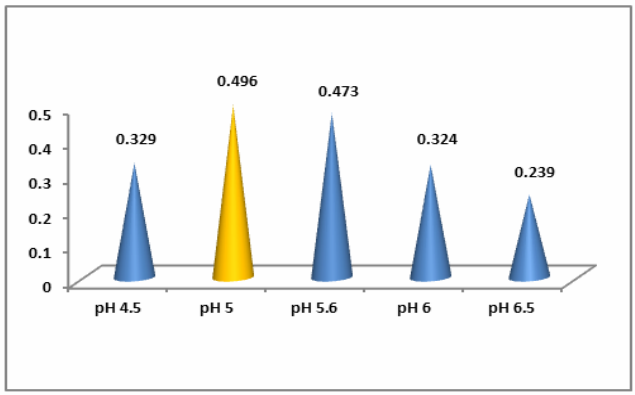


Figure (12) Dry biomass values of *A. terreus* MD10-3(0) By using different pH(s)

Temperature of incubation

To identify the effect of the temperature of incubation on VA production from the fungal isolate *A. terreus*, four different temperature degrees were experienced on the production media. The results shown in figure (13) and (14) display that the highest production of VA and the highest value for the dry biomass for the fungi were on the temperature 30 C of incubation. VA productivity reached 11.937 mg/ml while the dry biomass reached 0.58 mg/100ml. consequently, 30 C for incubation was adopted for all later stages of production. However, when the temperature goes higher, the production decreases as shown in the figures (13,14). This might be attributed to the formation of other acids like oxalic acid and the diminishing activity of the bioconversion enzyme (Vergano *et al.*, 1996).

Moreover, Dowdells *et al.* (2010) agreed on adopting 30 ºC for the production of Gluconic acid from *A. terreus* fungi. This temperature is also recommended and adopted by other researchers like Lesage- Meessen *et al.* (2000) and Mazhar *et al.* (2017). By considering these results, 30 C is recommended for the coming studies.

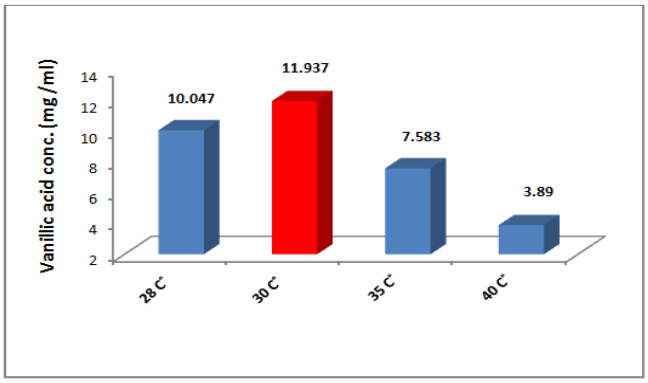


Figure (13) the effect of different temperature on VA Production by *Aspergillus terreus* MD10-3(0)

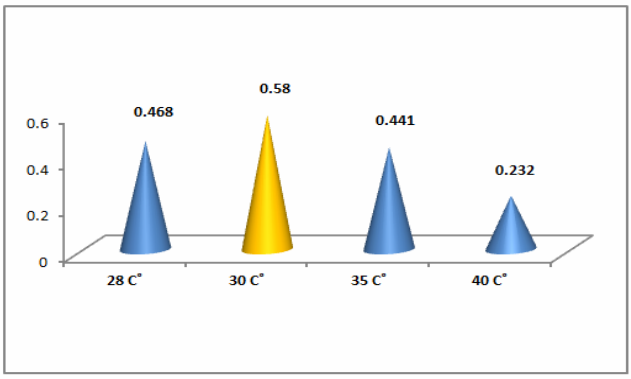


Figure (14) Dry biomass values of *A. terreus* MD10-3(0) by using different temperature

Determination of the best size of inoculum

Different levels of inoculum size were used for inoculating the production media. Figure (15) refers to the highest productivity for VA which occurred with the use of 1 × 10^6 cfu/ml inoculum size. Production of VA recorded 13.468 mg/ml. This inoculum size yielded the highest production of VA while the dry biomass for this inoculum size was not the highest value as displayed in figure (16).

The decreasing of VA production at 1 ×10^7 could be attributed to the crowded cells. This leads to slower and lower growth of fungi. The results of the present study differ from the results given by the researcher Lesage–Meessen *et al.* (1996), who used 2 × 10^5 cfu/ml inoculum size of *Aspergillus niger.*

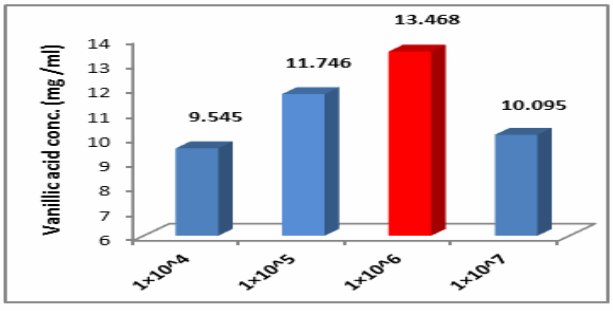
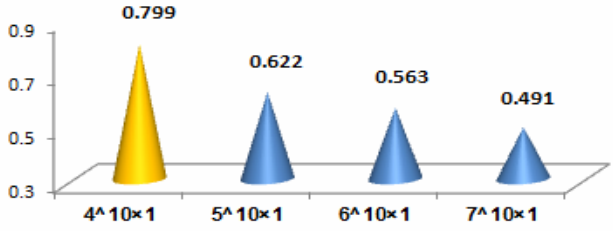


Figure (15) the effect of different incubation size on VA production by *A. terreus* MD-3(0)



Figure(16) dry biomass values of *A. terreus* MD10-3(0) by using different inoculum size

Effect of aeration

Figure (17) shows the effect of using different revolve per minute (rpm) during VA production. The highest production was recorded on 150 rpm/min. VA production reached 13.94 mg/ml. It is clear that the production goes higher in proportion with the number of rpm to reach 150 rpm/min which gave the highest value of production. The more rotational speed goes faster, the more the dissolved oxygen in the media increases. Consequently, the growth of fungi and its productivity increases in limited way and might decrease. Revolution speed has a very essential role in mixing the ingredients of the media which improves the ability of the growing fungi on bioconversion activities. Yet, when this speed goes higher than this range, it causes damage for the cells of the fungi when they clash with the walls of the container (Al- Mua'iny, 2017). This can be the same reason to explain the decrease of the dry biomass when rpm goes higher than 150 rpm/min as shown in the figure (18).

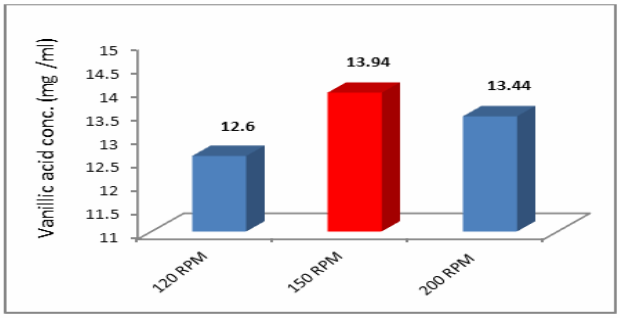


Figure (17) the effect of different rpm on VA Production by *Aspergillus terreus* MD10-3(0)

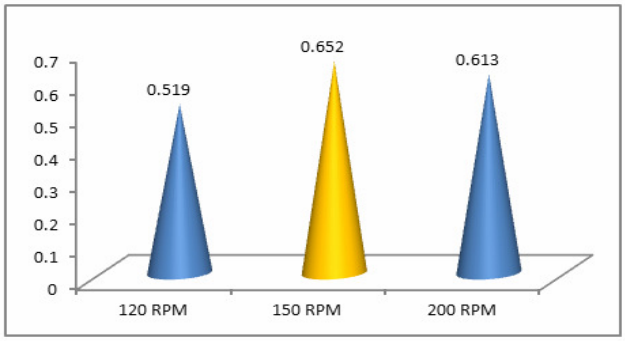


Figure (18) Dry biomass values of *A. terreus* MD10-3(0) by using different rpm(s)

The results of the present study for the best aeration for VA production agree with what was assured and adopted by each of Topakas *et al*. (2003) and Zheng *et al*. (2007). They used 150 rpm/min for VA production. However, Lesage – Meessen *et al*. (2000) used 120 rpm/ min for VA production from *A. niger*. This difference is related to the type of the fungi as well. 150 rpm/min is recommended for the coming studies.

Period of incubation

Figure (19) presents the effect of incubation period on VA production. It involved (6, 7, 9, 12 days) on 30 C. The best production yield was gained on the 7th day of incubation. VA production reached 14.9 mg/ml. This value was the highest one achieved during optimization process. The figure shows that there is a difference in the concentration of VA during incubation period. The concentration of production increased gradually to reach its highest value at the 7th day, and then it goes lower after that. This can be attributed to the formation of production inhibitors, and nutrients consumption along with the increase of incubation period more than 7 days.

Moreover, figure (20) clarifies the increasing and decreasing of dry biomass values along the different periods of incubation. It goes higher on the seventh day then it goes down and re-goes higher again on a longer period of incubation. This can be explained that the phenolic compounds concentration was low in the first few days of incubation because they are found in a chelating form naturally. Few of them can be found in a free form. Also, when the period of incubation goes longer than 7 days, phenolic acids are exposed for polymerization and the fungi can get exhausted (Bind *et al*., 2014).

Lesage-Meesson *et al*. (1996) adopted the 7th day four harvesting the production while Hung *et al*., (1993) adopted 10 days four VA production by *Rhodotorula rubra*. Other studies refer to that some yeasts need (24-48 h) four incubation and production. This certifies that incubation period basically depends on the type of microorganism exploited in production (Ashengroph and Amini 2017).

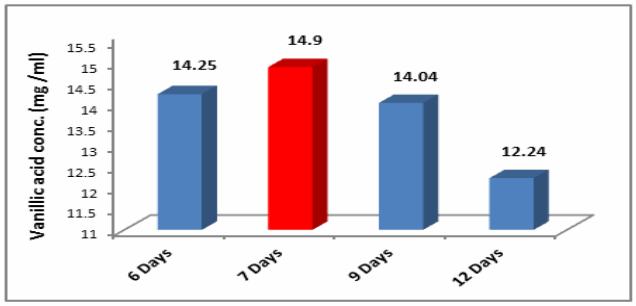


Figure (19) the effect of different periods of incubation On VA Production by *Aspergillus terreus* MD10-3(0)

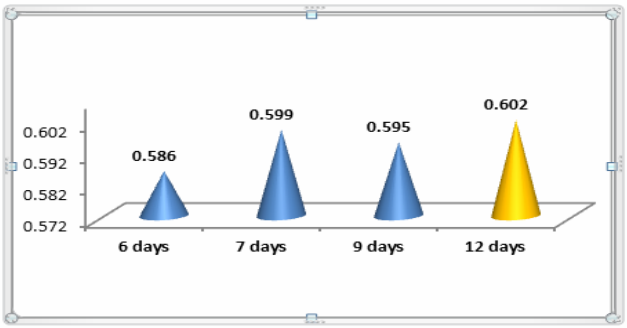


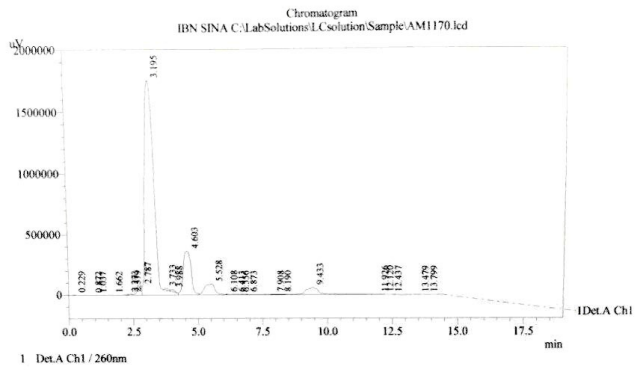
Figure (20) Dry biomass values of *A. terreus* MD10-3(0) By using different periods of incubation

HPLC analysis of VA production

This technique of chromatography is used to determine the quality and purity of VA by detecting the retention time by high performance liquid chromatography technique. The two figures (21) and (22) display the appearance of two absorption peaks for the standard VA solution and the sample under study with the retention time: 3.269 and 3.195 min respectively. This result reveals that the production solution is VA with high purity. Some studies refer to that the retention time of VA was 5.7 min which is near to what is found in the present study (Muti and Olimat, 2018). It worth mentioning that retention time can be affected by two important factors: the distance passed by the sample through the column and the speed in which the sample can pass this distance (Kupiec, 2004).

HPLC analysis is considered suitable in a perfect way to isolate and identify most of the compounds due to the variety and availability of the columns used. The apparatus has a high sensitivity and suitability to separate the non-volatile substances and non- tolerable substance for high temperature. HPLC is used greatly in various fields like pharmaceutical, food industrial, environmental and agricultural fields (Bucholz *et al*., 2000; Hao *et al*., 2007; Stanciu *et al*., 2008).

==== Shimadzu LCsolution GPC Analysis Report ====



PeakTable

Detector A Ch1 260nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.229	2701	164	0.005	0.007
2	0.872	1632	213	0.003	0.009
3	1.037	1931	158	0.003	0.007
4	1.662	6598	277	0.012	0.012
5	2.273	36431	6393	0.065	0.266
6	2.379	90957	8469	0.161	0.352
7	2.787	891263	75571	1.579	3.141
8	3.195	41858625	1757303	74.147	73.037
9	3.733	184970	14139	0.328	0.588
10	3.988	185570	15799	0.329	0.657
11	4.603	7682135	366713	13.608	15.241
12	5.528	3005630	90341	5.324	3.755
13	6.108	3924	574	0.007	0.024
14	6.413	8742	801	0.015	0.033
15	6.556	9677	912	0.017	0.038
16	6.873	2861	371	0.005	0.015
17	7.908	41283	1980	0.073	0.082
18	8.190	23660	1666	0.042	0.069
19	9.433	2403752	62839	4.258	2.612
20	11.926	4693	449	0.008	0.019
21	12.120	1897	349	0.003	0.015
22	12.437	1057	147	0.002	0.006
23	13.479	2584	256	0.005	0.011
24	13.799	1158	166	0.002	0.007
Total		56453730	2406051	100.000	100.000

Figure (22) Vanillic acid produced by *A. terreus*

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