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Assessment of Biochemical Changes of Four *Aspergillus* Species Grown on the Medium from Agricultural Wastes

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Abstract: Hazardous disposal of agricultural wastes (AW) has adverse environmental consequences, including water and air pollution and the potential for disease outbreaks. On the other hand, the utilization of AW represents a missed opportunity to harness a valuable economic resource. This study was conducted with the objective of utilizing a composite medium comprising agricultural waste to cultivate Aspergillus species and assessing its impact on the species' internal chemical composition compared to malt extract media (ME). Our findings demonstrate that the agricultural waste-based medium is abundant in essential nutrients, including soluble proteins and sugars, and is also enriched with a variety of secondary metabolites. Consequently, this Change in the growth medium induces changes in the physical characteristics of fungal biomass, such as color and texture, along with a high content of biomass proteins and secondary metabolites, including phenols, flavonoids, carotenoids, and antioxidants. The *A. avenaceous* gave the highest biomass (1.1412 ± 0.4 g), while the *A. niger* gave the highest value of proteins (16.06 ± 0.4 mg/g), phenols (33.37 ± 0.8 mg/g), flavonoids (4.84 ± 0.4 mg/g), carotenoids (1.131 ± 0.09 mg/g). *A. carbonarius* gave the highest value of antioxidants (IC_{50} = 0.28 ± 0.06 mg/mL). In contrast, using malt extract as a growth medium results in high carbohydrate and lipid production; *A. flavus* showed the highest value for fats (56.6 ± 0.9 mg/g), whereas *A. carbonarius* showed the highest value for sugars (167.1 ± 6.2 mg/g). Additionally, the malt extract medium contributed to low levels of secondary metabolites, which was offset by an increase in the protein bands of the fungal species. This research recommends the use of agricultural wastes to grow fungi species as an environmentally and economically important microbiological application.

Keywords: Agricultural waste, Medium, *Aspergillus*, Secondary metabolites.

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1. INTRODUCTION

Beyond the immediate concerns related to waste disposal and the mitigation of visual and olfactory nuisances, environmentalists are deeply preoccupied with the overarching issue of ecological damage affecting our forests, terrestrial landscapes, and crucial water resources. This concern is underscored by historical records, notably from 1967 and 1969, when agricultural practices emerged as the second most substantial source of pollution, primarily evidenced by significant fish mortality. Notably, the culpability did not predominantly rest with fertilizers but rather with insecticides and food production processes, which not only compromised water quality but also catalyzed the propagation of diseases within adjacent regions. This historical perspective amplifies the urgency of addressing these multifaceted environmental challenges and underscores the imperative of seeking more sustainable solutions (1).

A significant quantity of agricultural residues resulting from crop cultivation and food processing, including materials like straw, corncobs, and remnants from fruit and vegetable processing, is regularly produced. The methods used for managing, treating, and disposing of these agricultural wastes have the potential to negatively impact the quality of air, water, and soil. Unfortunately, a substantial portion of these waste materials is presently disposed of in landfills or released into rivers, thus causing notable environmental hazards (2).

Plant biomass serves as a fundamental feedstock for various industrial applications due to its richness in lignocellulosic materials and other valuable compounds. These resources can be effectively

harnessed through physical, chemical, and biotechnological treatments. As a result of these processes, a range of useful products can be derived, including extracts with applications in the food and pharmaceutical industries and platform chemicals such as organic acids produced through fermentation, biofuels, energy sources, and fiber residues suitable for use in bio-composite materials (3).

Biological waste treatment processes involve a blend of microorganisms with the ability to break down organic waste materials. Within certain bounds, these microorganisms can adapt to fluctuating organic loads and environmental factors like temperature and pH levels. However, the presence of extreme temperatures, elevated concentrations of metal ions, or toxic chemicals can impede or entirely inhibit their activity.

The composition of microorganisms in different biological treatment systems varies and can encompass bacteria, fungi, algae, protozoa, rotifers, Crustacea, bacteriophage, worms, and insect larvae, depending on the prevailing environmental conditions (4,5).

Fungi are truly remarkable organisms found in diverse ecosystems, exhibiting the ability to thrive on both living and deceased organic matter, including agricultural plant residues and municipal solid waste. Many fungi are adept at colonizing solid substrates, secreting extracellular enzymes to break them down, and subsequently reabsorbing the resulting nutrients through their fungal hyphae (6).

Aspergillus species are among the first fungal organisms cultivated on artificial media and studied for their biochemical properties. They inhabit a wide range of environments, with some being common saprophytes in the soil while others are found on stored food items (7). These species are known for producing a diverse array of secondary metabolites that are of industrial significance and therapeutic relevance, including antibiotics and lovastatin. As a result, they have garnered considerable attention within the scientific research community (8).

Given their filamentous nature, extrinsic factors such as water availability, temperature, pH levels, and the composition of the nutrient medium exert a substantial influence on the growth and biosynthesis of secondary metabolites in *Aspergillus* species (9).

In our research, we investigated the chemical composition of a specially formulated agricultural waste medium. We then examined how this medium affected the growth of *Aspergillus* species, their biomass production, and the physical characteristics of their hyphae. Furthermore, we assessed its impact on the efficiency of primary and secondary metabolite production, drawing comparisons with a commonly used commercial malt extract medium.

2. EXPERIMENTAL SECTION

2.1. Collection and Preparation of Agricultural Waste

Agricultural waste materials were collected, including potatoes, eggplant fruit, zucchini fruit, orange peels, corn peels, bean pod peels, bean leaves, and bean stems. To prepare these materials, they underwent a series of steps: first, they were thoroughly washed, followed by drying and chopping into smaller pieces. Subsequently, the chopped materials were allowed to air-dry in the shade. Once sufficiently dried, samples were finely ground using an electric mixer, sieved to ensure uniformity, and then stored in plastic bags at room temperature in a dry environment for future use (10).

2.2. Chemical Analysis and Preparation of Agricultural Waste Medium

The agricultural waste (AW) medium was meticulously prepared as follows: 2 grams of each plant powder were added to 500 mL of distilled water, and the mixture was then sterilized using an Autoclave. After sterilization, the medium underwent filtration, and its pH was carefully measured and adjusted to 5.6 before undergoing another round of sterilization. To determine the protein content of the medium, the Bradford assay method was used by the Coomassie blue detector; the bovine serum albumin was employed to make the standard curve (11). The phenol sulfuric acid method was employed for the assessment of soluble sugars, as described by Dubois et al. in 1956. The glucose equivalent was utilized to establish the standard curve for this purpose (12).

The total phenol content was quantified by adding 1 mL of medium broth to 0.5 mL of 50% Folin-Ciocalteu and 1.5 mL of 20% Na_2CO_3, with the final volume adjusted to 5 mL using distilled water (13). The samples were then incubated in darkness for one hour, and their absorbance was subsequently measured at 760 nanometers. The gallic acid equivalent was employed to construct the standard curve.

Lastly, the concentration of dissolved salts was determined using a pH meter. For comparative purposes, the same analytical procedures were applied to the Malt (M) medium broth (14).

2.3. Fungal Strains Used

The *Aspergillus* strains employed in this study were originally sourced from the soil of pine forests in Syria. 1 g of the soil was diluted in 99 mL distilled sterile water. Several dilutions were prepared to get ^{5}10 concentrations. 1 mL of ^{5}10 solution was grown on a potato dextrose agar (PDA) petri dish and incubated at 28°C for three days. This process was repeated several times. The genus *Aspergillus* was verified based on colony characteristics and microscopic characteristics. These strains underwent a purification process and were ultimately cultivated Malt extract agar and subjected to an identification protocol as per established methods (15,16).

2.4. Preparation of Fungi Culture

The *Aspergillus* species were grown on PDA for about one week at 28 ± 2 °C. The spore suspension was collected in tween 0.05% and was enumerated by a qualified hemocytometer.

1 mL spore suspension of each fungal strain (10^6 spores/mL) was added to 1 L of agricultural waste broth medium and malt extract broth medium (17).

The medium was put at 28 °C while shaking at 150 rpm for three days. The Fungal biomass was separated, water-washed, and oven-dried at 100 °C until constant weight had been reached. Dry Fungal biomass was crushed in a mortar and then Stored in a −80°C freezer (18).

2.5. Preparation and Extraction of Fungal Metabolites

500 mL of 80% methanol was Added to fungal biomass and sonicated in an ultrasonic bath sonicator for 180 min. After that, the samples were stirred vigorously for two days by using a magnetic stir bar and magnetic stirrer. The extracts were filtered with vacuum filtration and evaporated in a rotary evaporator, then put into a pre-weighed 20 mL glass vial. The weight of the dried samples was obtained using a scale to calculate the yield in mg. The dried extract was stored in a −20 °C freezer (19).

2.6. Determination of Total Sugar (Carbohydrate) and Total Phenols in Fungal Extract

The carbohydrate content was determined by the phenol sulphuric acid method, and the phenol content was determined by the Folin-Ciocalteu method.

2.7. Determination of Total Flavonoids in Fungal Extract

The TFC of the fungal extracts was determined by an aluminum chloride colorimetric assay. 500 µL of methanolic extract 80% was added to 150 µL of $NaNo_3$ 5% and incubated in the dark for 5 minutes. One hundred fifty microliters of $AlCl_3$ 10% were added to the extract and incubated in the dark for 6 minutes. 1 mL of NaOH 1M was added and incubated for 15 minutes in the dark. The absorbance of each mixture was determined at 510 nm. Quercetin equivalent was employed to make the standard curve (20).

2.8. Determination of Total Carotenoids in Fungal Extract

The fungal extract was diluted in methanol absolute to get a concentration of 1 mg/mL; chlorophyll a, chlorophyll b, and total carotenoids were determined by the following equation using a spectrophotometer resolution range 1-4 nm:

$$C_a = 15.65\ A_{666} - 7.34\ A_{653} \quad \text{(Eq. 1)}$$

$$C_b = 27.05\ A_{653} - 11.21\ A_{666} \quad \text{(Eq. 2)}$$

$$C_{x+c} = (1000\ A_{470} - 2.86\ C_a - 129.2\ C_b)/221 \quad \text{(Eq. 3)}$$

C_a is total chlorophyll a, C_b is total chlorophyll b, and C_{x+c} is total carotenoids by concentration µg/mL (21).

2.9. Determination of DPPH Radical-scavenging Activity in Fungal Extract

A series of the different concentrations (0.1 – 2 mg/mL) of each methanolic extract 80% was prepared, and 200 µL of the fungal extract was added to 2 mL of 1, 1- diphenyl-2-picryl hydrazyl (DPPH) reagent. The samples were incubated in the dark for 30 minutes; the absorbance was measured at 517 nm. The ability to scavenge the DPPH radical was calculated using the following equation: DPPH scavenging effect (%) = ((A0-A1/A0) × 100), where A0 was the absorbance of the control reaction, and A1 was the absorbance in the presence of the sample. The percentage of scavenging activity obtained was subsequently plotted against the sample concentration. The IC_{50} value, defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%, was calculated from the results and used for comparison. A lower value of IC_{50} indicates a higher efficiency of scavenging free radicals. The results were compared to Ascorbic acid (Vitamin C), which was prepared as standard with different concentrations (0.01, 0.02, 0.03, 0.04, 0.05 mg/mL) (22).

2.10. Preparing of Crude Extracts and Measuring of Protein Values

The Tris-HCl 6.8 0.5 M with phenylmethylsulphonyl fluoride (PMSF) 0.5 mM was prepared. 1 mL of this solution was added to 0.5 g of fungal biomass. Then, the biomass was crushed in a mortar. The crude extracts were separated from the cell walls, remaining by centrifugation at 25000 rpm for 30 min, and then were processed through three stages. The protein content was determined using Bradford's method. The bovine serum albumin was employed to make the standard curve. The supernatants were kept in microtubes in a −20 °C freezer (23).

2.11. Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis

The fungal protein extracts were analyzed using the SDS-PAGE method with %13 separating gel and %4 stacking gel (Sigma) at a constant voltage of 90 V for one hour and 120 V for two hours.

The extracts were boiled for 5 min with a loading buffer containing 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol, 0.004% Bromophenol Blue, and 0.125 mol/L Tris–HCl 6.8.

35 µL of each sample was loaded on a gel. Along with the standard protein marker (blue plus, trance. China). The gel was stained with 0.25% Coomassie Brilliant blue R 250 (Sigma) (24).

2.12. Determination of Total Lipids

The fungal biomass was prepared for extraction by grinding the dried material into a powder using a mill. For hexane extraction, 300 mL of n-hexane was added to 4 grams of fungal biomass powder. Conical flasks were sealed with aluminum foil to prevent solvent evaporation. Then, all extraction mixtures

were subjected to one hour of sonication using an ultrasonic bath sonicator. Following sonication, they were agitated at 200 rotations per minute at room temperature for 24 hours.
To remove cellular residue, the mixture was centrifuged at 25,000 rotations per minute for 30 minutes. The hexane phase was collected and evaporated in a rotary evaporator, and each extract was collected in a pre-weighed flask. The flask was subsequently heated to dryness in an oven at 60°C to enable gravimetric quantification of the lipid extract.

The resulting crude lipid was re-dissolved in hexane and transferred into a sealed glass vial for storage in a −20 °C freezer (25).

2.13. Statistical Analyses

Data were analyzed using the IBM SPSS Statistics software (Version 22.0)—the experimental design involved four groups. To investigate the effects of two factors (independent variables) and their interaction with the dependent variable, a two-way Analysis of Variance (ANOVA) was performed. This statistical approach is ideal for understanding how each factor contributes to the observed outcomes and whether their effects are independent or interactive. Results were deemed statistically significant at a p-value less than 0.05. All data are reported as means ± standard deviations (SDs).

3. RESULTS AND DISCUSSION

The agricultural waste medium exhibited a dark brown color with a pH of 5.8, while the malt extract medium was a transparent yellow with a pH of 5.5. The agricultural medium was rich in nutrients, containing significant amounts of soluble proteins and sugars, as well as a high concentration of salts and plant-derived secondary metabolites such as phenols (see Table 1) (26). In contrast, the malt medium had slightly higher levels of soluble sugars and proteins but lower concentrations of salts and phenols (Table 1). Statistical analysis revealed significant differences in the average chemical compositions of the two media ($p < 0.001$).

These variations in medium composition influenced the growth characteristics of the fungi. Four fungal species were identified through macroscopic observations (colony diameter and color) and microscopic examinations (vesicle, metula, phialides, and conidia shapes and sizes): *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus carbonarius*, and *Aspergillus avenaceous* (16). Both media supported robust fungal growth, resulting in significant biomass production with noticeable differences in color and texture (27). The fungi on malt medium generally showed light, creamy colors with a cohesive, rubbery texture, whereas those on agricultural waste were darker and more fragile (see Figure 1).

Table 1: Chemical composition of the tested media.

Medium g/L	Protein	Sugars soluble	Phenols	Dissolved salts (µc/cm)
ME	0.17 ± 0.01	2.9 ± 0.2	0.69 ± 0.09	507 ± 12
AW	0.10 ± 0.02	2.2 ± 0.1	1.4 ± 0.05	915 ± 2

Notably, the agricultural waste medium appeared to promote high biomass production, coinciding with a low in stored fat. This phenomenon can be attributed to fungi primarily utilizing carbon from the medium for cellular growth and repair, with the surplus being allocated to fat storage (28). Among the species studied, *A. avenaceous* displayed the highest biomass production, with values of 1.1412 ± 0.09 g and 0.8261 ± 0.06 g on the agricultural waste and malt extract media, respectively. Statistical analysis indicated significant differences in mean biomass between the species across the two media.

The dark brown color of the mycelium is attributed to the presence of pigments like carotenoids (see Table 2). The fragility of the mycelium may result from the medium's high salt content. The high salt prompts the fungi to develop thin cell walls with greater porosity, facilitating exchange processes with the environment (29-31).

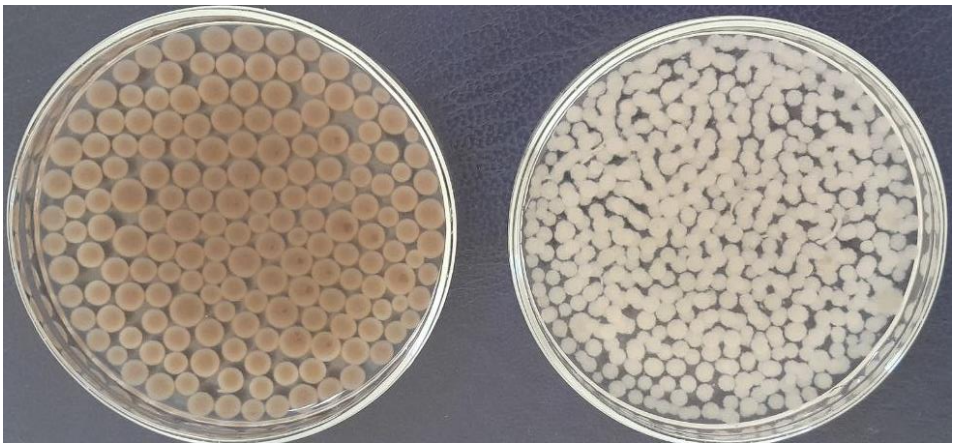

Figure 1: The Biomass of *A. niger* on agricultural waste and malt extract media, in order from the left.

The chemical composition of the medium significantly impacted lipid and carbohydrate synthesis in the fungal biomass (32). Malt medium, being richer in nutrients, showed slightly higher lipid and carbohydrate levels. Although total lipid content showed no significant variation among individuals, *A. flavus* recorded the highest production in both media (47.4 ± 0.2 - 56.6 ± 0.9 mg/g; Table 2), aligning with findings from similar studies (33). In terms of carbohydrates, *A. carbonarius* produced the most in malt extract, measuring 167.1 ± 6.2 mg/g (Table 2), a finding consistent with other reports (34). Statistical analysis confirmed significant differences in the average total lipids and carbohydrates between species on both media at p-value=0.014 and p-value<0.001, respectively.

Table 2: The Basic chemical composition of fungal species on the tested media.

	Biomass (g)		**Proteins (mg/g)**		**Lipids (mg/g)**		**Carbohydrates (mg/g)**	
Medium	ME	AW	ME	AW	ME	AW	ME	AW
A. niger	0.434 ± 0.08	0.544 ± 0.04	2.247 ± 0.3	16.06 ± 0.4	24.2 ± 0.5	14.7 ± 0.3	84.21 ± 7.1	29.17 ± 4
A. carbonarius	0.1852 ± 0.06	0.5083 ± 0.03	3.483 ± 0.3	15.169 ± 0.3	13.4 ± 0.6	11.7 ± 0.3	167.1 ± 6.2	57.86 ± 4
A. flavus	0.365 ± 0.04	0.5211 ± 0.06	1.573 ± 0.1	4.045 ± 0.2	56.6 ± 0.9	47.4 ± 0.2	52.69 ± 5.5	48.55 ± 5.6
A. avenaceous	0.8261 ± 0.06	1.1412 ± 0.09	4.270 ± 0.2	4.494 ± 0.3	24.7 ± 0.5	19.3 ± 0.3	103.52 ± 3	60.83 ± 2

In contrast, the agricultural waste medium directed the species to synthesize high levels of proteins, phenols, flavonoids, and carotenoids (Table 3), which are antioxidant compounds and anti-stress factors. In fact, fungi use these compounds for cellular construction and repair and to resist pressure factors (9, 35).

Protein production differed between species in both media. The best species producing proteins on the agricultural waste medium was *A. niger* 16.06 ± 0.4 mg/g. The species that was not affected by changing the medium is *A. avenaceous*, which also was the best in producing protein on the malt extract medium (Table 2). These results are comparable to those obtained in similar studies (36).

The production of phenols and flavonoids, and carotenoids differed among the species on agricultural waste medium, and the best species in production was A. niger by 33.37 ± 0.8 mg/g – 4.84 ± 0.42 mg/g – 1.131 ± 0.09 µg/g for phenols, flavonoids, and carotenoids respectively. The results are coherent with other studies (37-39).

The species grown on the agricultural waste medium (except for *A. flavus*) displayed lower IC_{50} values, revealing a higher antioxidant activity; these species also exhibited greater phenol levels compared to those grown on malt medium (Table 3). The species showing the highest antioxidant activity was *A. carbonarius*, with a half-maximum inhibitory concentration of 0.28 ± 0.06 mg/mL (Table 3). These results agreed with those of previous studies (20,40). The averages of the total proteins, phenols, flavonoids, and carotenoids between the species on both media were significantly different at p-value<0.001.

Table 3: The Secondary chemical composition of fungal species on the tested media.

	Phenols mg/g		**Flavonoids mg/g**		**Carotenoids µg/g**		**IC_{50} of antioxidant activity mg/mL**	
Medium	ME	AW	ME	AW	ME	AW	ME	AW
A. niger	18.70 ± 0.2	33.37 ± 0.8	0.27 ± 0.03	4.84 ± 0.4	0.276 ± 0.05	1.131 ± 0.09	1.42 ± 0.09	0.38 ± 0.04
A. carbonarius	13.86 ± 0.4	29.84 ± 0.4	0.33 ± 0.02	4.02 ± 0.3	0.134 ± 0.03	0.797 ± 0.06	0.47 ± 0.05	0.28 ± 0.06
A. flavus	11.67 ± 0.3	11.19 ± 0.5	0.17 ± 0.05	1.51 ± 0.3	0.294 ± 0.04	0.418 ± 0.05	0.87 ± 0.04	1.47 ± 0.03
A. avenaceous	10.3 ± 0.3	13.51 ± 0.3	0.51 ± 0.1	1.73 ± 0.2	0.227 ± 0.04	0.426 ± 0.05	0.77 ± 0.07	0.63 ± 0.04

(Ascorbic acid IC_{50}: 0.0467 mg/mL = 0.00026 mM/mL)

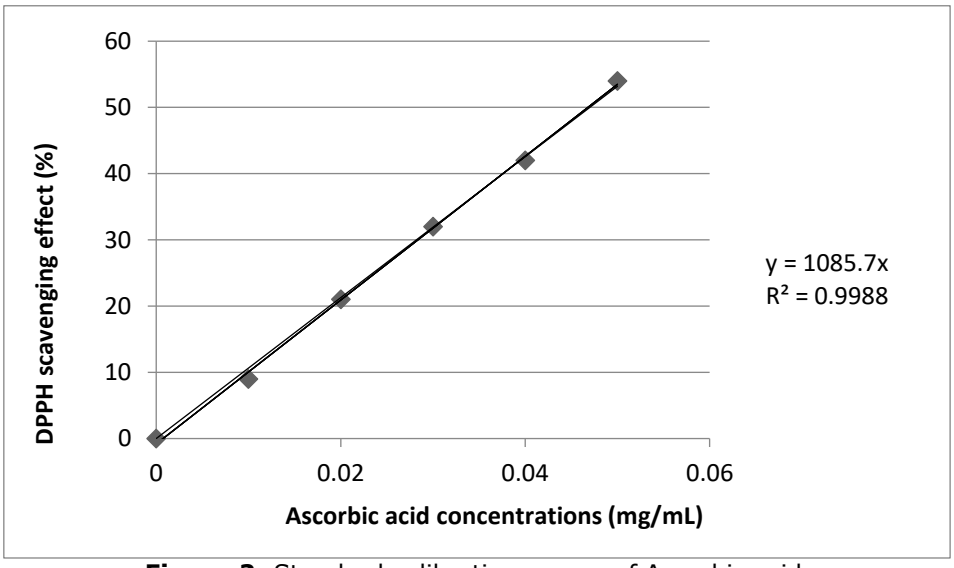

Figure 2: Standard calibration curves of Ascorbic acid.

The qualitative Electrophoretic protein pattern for every species was different after cultivating them on malt extract medium and agricultural waste medium.

The highest number of protein bands for the species on the malt extract medium and agricultural waste medium was 20 and 18 bands for *A. flavus*, respectively. A. carbonarius exhibited the lowest number of protein bands on both media, 12 and 10 bands, respectively. In general, the high number of protein bands in the species was associated with low production of phenols, flavonoids, and antioxidant capacity.

The resulting bands could be stress proteins or antioxidant enzyme protein groups produced by the fungi to improve their adaptability to the medium (41-43). *A. avenaceous* with the same number of bands for both media shows a small change in the efficiency of its production of phenols and flavonoids (Table 3-4).

Table 4: The frequency of protein bands obtained from *Aspergillus* species (kilodalton) on the tested media.

Species	***A. niger***		***A. carbonarius***		***A. flavus***		***A. avenaceous***	
Medium	ME	AW	ME	AW	ME	AW	ME	AW
Proteins Bands Number	14	10	12	10	20	18	17	17
190		+			+	+	+	+
114		+			+	+	+	+
112	+		+	+				
111.8	+			+	+	+	+	+
89.4			+		+	+		
83.8				+				
82.9							+	+
80.3							+	+
78.2	+		+		+	+		
77.8	+							
70						+	+	
67.4	+	+					+	+
67.1			+	+				
64.8					+			
62.4	+				+		+	+
59.4	+	+			+			
56.5					+			
55						+	+	+
52	+							
50.5			+	+	+			
46.6			+		+	+		+
44.8	+							
43							+	+
42.1			+	+				
41.2		+			++	+		
38.5					++	+		
37.6							+	+
36.7							+	+
34.1	+	+	+	+				
31.9							+	+
28.7			+		+	+		
28							+	+
26.7	+	+	+	+				
25.3	+	+	+	+	+	+		
24.3							+	+
18.5					+	+		
16.2					+	+		
15.4					+	+	+	+
10.7	+	+			+	+	+	+
10	+	+	+	+	+			

4. CONCLUSION

Our findings highlight the potential utilization of agricultural waste, previously considered detrimental to the environment and economically wasted. By formulating a medium enriched with soluble proteins and sugars, we demonstrated its suitability for fostering the growth of *Aspergillus* species. This medium also induced distinctive physical attributes, including color and texture, in addition to boosting biomass, protein content, and the production of secondary metabolites, particularly phenols, due to the stress factors introduced during cultivation, such as high salt and phenol concentrations.

Conversely, the use of malt extract medium was associated with high primary compounds such as carbohydrates and lipids.

5. CONFLICT OF INTEREST

There is no conflict of interest with any institution.

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