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GROWTH MEDIA FROM PLANT EXTRACTS FOR INDUSTRIALLY IMPORTANT FUNGUS; *ASPERGILLUS NIGER*

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Abstract

The current study is performed to assess growth supporting capability of aqueous plant-based extracts which were further supplemented with sugarcane peel extract; SPEX and mineral salt solution (MSS) to formulate microbiological growth media for fungi particularly *Aspergillus niger.* Dry cell weight (DCW), extracellular proteins, reducing sugars, pH alteration in extract media (EM) was observed and compared with that of potato dextrose broth (PDB). The production of citric acid and enzymes (amylase/cellulase; endogluconase) were also assessed on preferred EM combinations. Highest DCW was found in coconut bark extract + SPEX + MSS; CBSM, Nimtree Bark + SPEX; NBS and date bark extract + SPEX + MSS; DBSM (0.207, 0.16 and 0.2 gram/ 25 ml) respectively with SPEX 30 % (v/v) in comparison to that of PDB (0.155 gram/ 25 ml) on day 4. The pH of EM was acidic with 2.81; the lowest in NBS with SPEX 30% (v/v). Reducing sugars were detectable in EM containing SPEX 30 and 20% (v/v) concentration. Protein content was maximum in NBS, DBSM and CBSM (33, 24.75 and 22.44 µg/ml) respectively with various SPEX concentrations on day 4 as compared to that in PDB (20.75 µg/ml). NBS with 30% (v/v) SPEX showed highest concentration of citric acid (7.33 g/l) whereas, amylase/endoglucanase activity was better in DBEX + MSS (0.2 and 0.0283 IU /ml) on day 3 and 7 respectively. It can be concluded from the above results that the EM combinations have been found to be suitable for growth and other activities of *Aspergillus niger*.

Key words: Dry cell weight, Reducing sugars, Protein, Biomass, Cellulase, Amylase.

Introduction

A wide range of microbiological cultivation media are commercially available in dehydrated form to study the growth, morphological/ metabolic characteristics of various fungi. The culture media means a suitable balance of nutrients providing similar environments to the naturally occurring cells subsequently allowing a proper metabolic activity (Gómez & Batista, 2006; Morales-Borrell *et al*., 2020).

However, the raised cost of these media has not only demonstrated to be an obstacle in the availability but their ever-elevated costs throughout the world has become a disturbing issue as well. In this regard major challenges are met by the industries where certain enzymes, metabolites and fungal biomass are needed to be acquired at a larger scale. A producer fungal species needs to be cultured in specialized vessels (fermenters) is constantly provided with nutrient materials. The industries usually prefer low-cost substrates as nutrients so that the final cost of the product may also become affordable for the end user. Furthermore, the areas in which Microbiology is applicable or requires application face similar constraints. Typically related with the study of growth and further characteristics of microorganisms in academic institutions, research organizations or diagnostic laboratories require use of expensive dehydrated culture media. On the other hand, large microbial inocula are required particularly in various industries, in the broad area of environmental biotechnology for environmental cleanup (bioremediation processes), efficient bio-fuel production and development of bio-fertilizers and bio-control agents for the organic and sustainable agriculture.

The problem related to the high cost of conventional microbiological culture media can be highlighted by taking an example of a medium which is generally used for the cultivation and isolation of fungi in the laboratories; Sabouraud's dextrose agar (SDA). The medium is being supplied and manufactured by well-known companies at a cost of more than PKR 23000 ($ 81) / 500 gram. The developing countries with the passage of time are facing severe economic crisis, unusual high inflation rates and hike in dollar prices, therefore, further rise in the existing charges are expected. In order to avoid these problems, we have no option left except to switch on to the products, which are economical (low cost), simple, readily available and easily accessible. Therefore, the formulation and development of media that may be prepared from easily accessible, cost effective as well as simple ingredients, may probably be the solution to resolve current problem. Preliminary studies carried out in our laboratory have clearly revealed the potential of plant-based extracts to be used as microbiological culture media (Saleem & Ali, 2017). Various plant extracts have been found to possess growth supporting ability for a wide variety of fungi including yeasts and molds (Saleem, 2017).

The fungi, being versatile and heterotrophic in nature are capable to produce an array of enzymes that contribute to transformation and stabilization of materials. Due to the higher carbon to nitrogen ratio in the fungal biomass, the efficiency to utilize carbon is greatly increased (Li *et al*., 2021; Karhu *et al*., 2022). The fungi have simple nutritional requirements as compared to bacteria. This includes the carbon source (monosaccharide; glucose), nitrogen (ammonium/glutamine), trace minerals, and some growth factors such as vitamins (van Nieuwenhuijzen *et al*., 2019; Gulmez *et al*., 2022).

In the laboratories, the media containing natural ingredients are routinely used for the cultivation of the majority of the fungi. One such medium is the potato dextrose medium which contains potato extract along with 2 % (w/v) of the glucose. Nevertheless, the concentration of the carbon source is more than sufficient in the medium. Therefore, keeping in view the minimum nutritional requirement of fungi, various culture media were developed containing a preferred variety of plant extracts

as the main ingredient. *Aspergillus niger* was particularly chosen to evaluate the suitability of formulated extract media combinations. *Aspergillus niger* is generally regarded as safe (GRAS) organism (dos Santos Nascimento *et al*., 2022) and it is an established industrial organism to produce organic acids and enzymes because of its natural ability (Upton *et al*., 2020).

Though, not much work has been done on growth enhancing property of plant extracts, plus major research findings are based to investigate the microbial growth inhibiting ability of the plant-based extracts. This is due to the fact that these tiny creatures have been involved in causing several ailments and negative impacts on the health of humans, animals or even plants ever since their discovery. Nevertheless, despite being microbi-toxic or microbicidal in nature, these extracts have exhibited to possess growth enhancing property for fungi, if used in lower concentration (Mahmoud *et al*., 2011). Furthermore, the studies performed to determine chemical composition of extracts obtained from various plant sources indicate that these extracts are rich in nutrients. One of the common constituents namely Glycosides or carbohydrates as reducing sugar have been found to be detected in this extract. For example, glycosides were found to be present when the extraction of *Bougainvillea* sp bracts, leaves/ flowers, nimtree bark and leaves was performed (Haggag & Elhaw 2022; Ohalete & Anyanwu, 2023). Similarly, the phytochemical property assessment of the extracts obtained from date bark or other components contained carbohydrates, glycosides and further chemical compounds as indicated by Ahmed *et al*., 2016.

Additionally, phytochemical studies done to evaluate the chemical composition in water-based coconut bark extracts are suggestive of the presence of glycosides and carbohydrates (Sivakumar *et al*., 2011). Chemically, glycosides contain carbohydrate; glucose and a non-carbohydrate component; genin or aglycone (Ajobiewe *et al*., 2020). These are among the group of organic substances containing a constituent sugar linked via glycosidic bond to a functional group (Obla, 2013). As defined by IUPAC (http://www.iupac.org/) all polysaccharides and disaccharides are considered as glycosides. Therefore, it is convenient to understand that upon their hydrolysis simple sugars are liberated. Thus, the aqueous medicinal and edible plant-based extracts may contain a considerable concentration of carbon containing substances.

Apart from sugar containing molecules, studies also reveal that certain phytochemical components commonly occur in these extracts e.g. saponins, tannins, flavonoids, alkaloids and steroids. Due to provision of an array of degradative enzymes these molecules may be used as a source of nutrient by the fungi. For example, *Aspergillus* species have the potential to degrade the tannic acid (a type of tannin) by producing tannic acid specific esterases thus releasing the residues of gallic acid from tannic acid in the medium (Arentshorst *et al*., 2021). This example suggests the enzymatic potential of fungi for the degradation of variety of substrates. Further studies reveal that the other phytochemicals are processed to be transformed into less harmful substances or consumed as a single nutrient source by fungi. Therefore, considering all these findings, this study was performed to determine the fungal potential for utilization of these materials as growth supporting agents when being present as the basal ingredient of microbiological culture media. In this regard several growth assays were performed not only to evaluate the efficacy of these laboratory formulated low-cost media, but the enzyme activity and production of metabolite was also compared with that on conventional media.

Material and Methods

Details regarding obtaining the plants and their components for preparation of stock extracts 20 % (w/v), use of sugarcane peel extract (SPEX) and media preparation was performed as mentioned by Saleem, 2017 (Table 1).

Choice of best possible extract media to cultivate *A. niger*: The choice of best suitable extract-based media was dependent on maximal radial expansion in minimum incubation period and number of spores counted in comparison to potato dextrose agar (Saleem & Ali, 2017) the best formulations of extract-based media were chosen to study further growth parameters of *A. niger*. These combinations are inclusive of NBS, DBSM, CBSM also BVSM (bougainvillea branch extract+SPEX+MSS).

The preparation of potato dextrose broth (PDB) was done according to the standard protocols whereas, the preparation of spore suspension was carried out as mentioned by Jernejc (2004) and Naqvi *et al*., (2013) and Hayer (2014).

Table 1. Preparation of liquid extract media (EM) combinations, Plant extract working concentration 2.5 % (v/v), SPEX working concentration 10 % (v/v). The other concentrations of SPEX [20 and 30% (v/v) were adjusted according to the concentration of stock].

Name of medium	Abbreviated for	From Stock plant extract (20% w/v) (ml)	From stock Sugarcane peel extract (25 % w/v) (ml)	Mineral salt solution (ml)	Distilled water (ml)	Total volume (ml)
BVSM	Bougainvillea branch extract + Sugarcane peel extract + Mineral Salt solution	125	400	475	-	1000
CBSM	Coconut bark extract + Sugarcane peel extract + Mineral Salt solution	125	400	475	-	1000
DBSM	Date bark extract + Sugarcane peel extract + Mineral Salt solution	125	400	475	-	1000
NBS	Nimtree bark extract + Sugarcane peel extract	125	400	-	475	1000

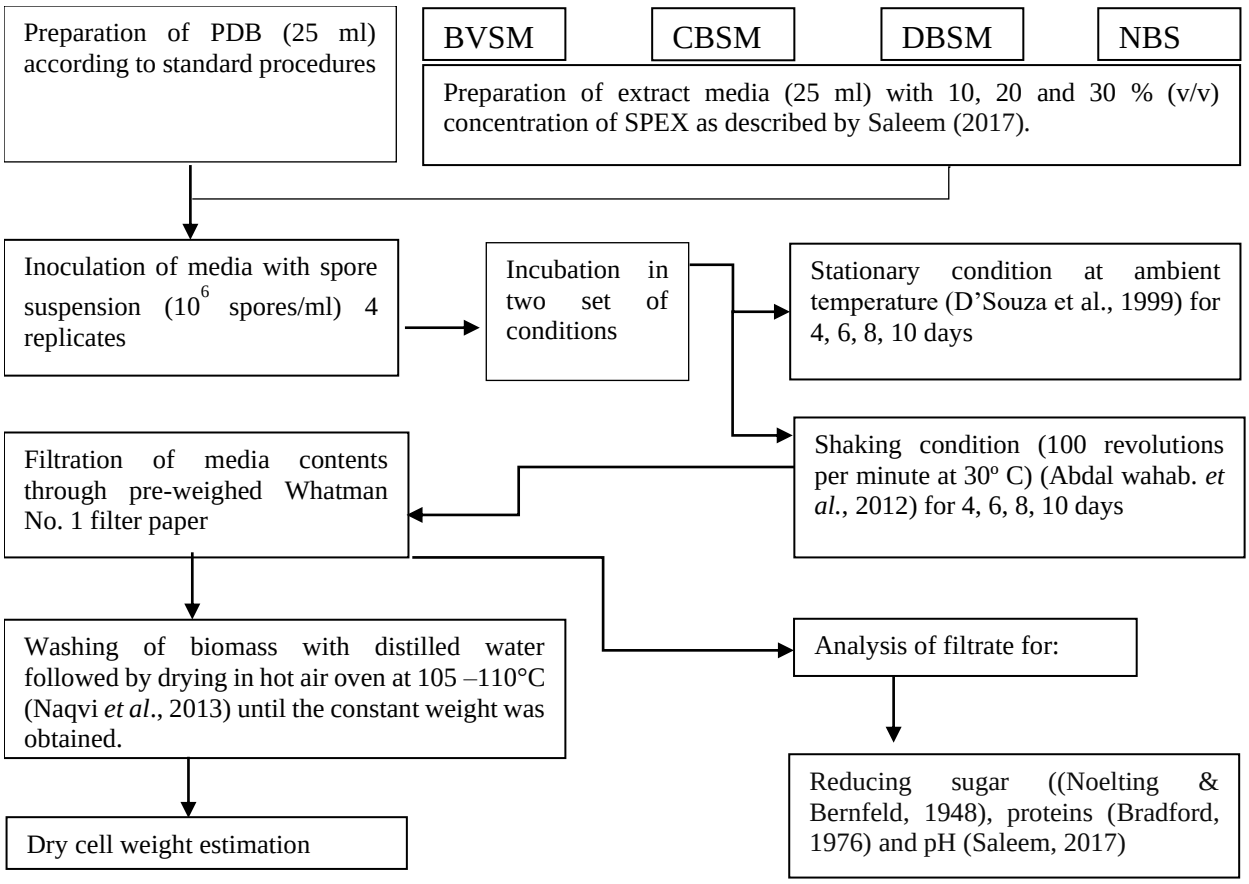

Fig. 1. Flow sheet showing methodology to perform growth assays of *A. niger* on preferred liquid extract media combinations.

Impact of varying concentration of sugarcane peel extract; SPEX on dry cell weight (DCW), protein, reducing sugar, and pH of medium: The chosen extract media combinations were supplemented with various SPEX concentrations to assess its impact on biomass, protein, reducing sugar production as well as the alteration in pH of media. The methodology was followed as mentioned in (Fig. 1).

Production of citric acid on NBEX along with varying SPEX concentrations was determined by using method (Anon., 1995; Kareem & Banjo, 2015). Whereas the methods for qualitative and quantitative cellulase/ amylase assays were followed as mentioned by Kasana *et al*., (2008) and Acharya *et al*., (2008) and Oshoma *et al*., (2010).

Result and Discussion

The highest dry cell weight production was observed at day 4 and 6 respectively on CBSM and NBS followed by sudden drop in the biomass. The lowest growth with regard to dry cell weight (DCW) in BVSM was observed in shaking cultures on 4^{th} day as compared to other extract media (Fig. 2). The biomass in DBSM was equivalent to the biomass on PBD till day 10. The P values also show moderately significant growth differentiation on NBS and BVSM at 4^{th} and 6^{th} day respectively in comparison to PDB. Significantly high values in NBS, CBSM and BVSM with 10 % (v/v) SPEX were observed at day 8 and 10 (Fig. 2). The biomass on NBS, DBSM plus BVSM with SPEX 20 % (v/v) was found to be as effective as PDB whereas raised in CBSM (p=<0.001) on 4^{th} day (Fig. 4). Highest DCW after 6 days of incubation was observed in NBS and CBSM 0.163 and 0.185g/25 ml respectively.

The stationary cultures of NBS and CBSM showed DCW values equivalent to PDB and low values in BVSM (Figs. 3,5 and 7). A significant increase (p=0.017) in DCW was observed on DBSM with SPEX 10% (v/v) at 4^{th} day which became similar as that in PDB at day 6 / 8 but declined at 10^{th} day. The plant extract-based media having SPEX 30 and 20 % (v/v) concentrations presented greater DCW values in comparison to the values of PDB in stationary cultures. The highest DCW; 0.283 g/25 ml was noted in DBSM with SPEX 20 % at 10^{th} day that is significantly higher (p=0.003) in comparison to that in PDB (0.235 g/25 ml). In other extract-based media combinations, DCW was significantly raised or else noted to be equivalent to that of PDB. In extract media, CBSM, NBS and DBSM having SPEX 30 % (v/v) the DCW values; 0.3, 0.275 and 0.27 g/25 ml respectively were achieved at day 6 (Fig. 7) which was raised significantly as compared to that of PDB; 0.19 g/25 ml. Regarding growth in shaking plus stationary conditions, Vasantha *et al*., (2014) stated that the production of biomass by *Aspergillus niger* was found to be higher during agitation. However, the studies between stationary and shaking cultures performed in our lab are suggestive of combined effect on DCW of *A. niger*. DCW in stationary cultures of PDB was found to be better throughout incubation period whereas, CBSM with 10 % SPEX had greater DCW values in shaking cultures than stationary cultures at 4^{th} day (p=0.007). Generally, dry cell weight in static cultures with extract-based media having SPEX 30 or 20 % (v/v) concentrations produced better results than in shaking cultures.

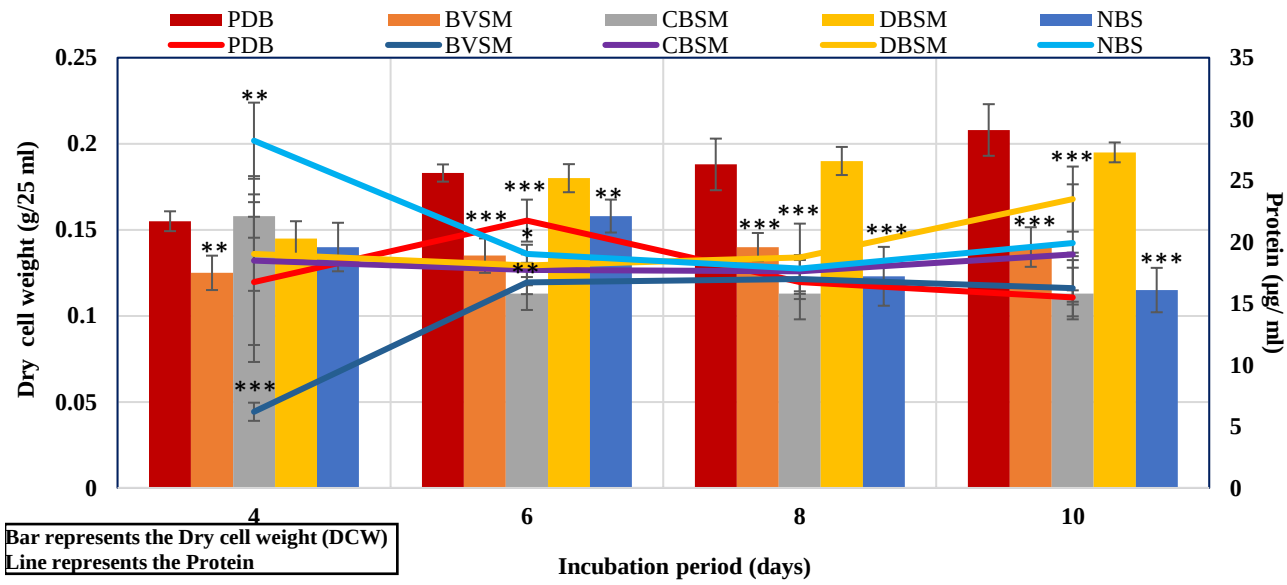

Fig. 2. Assessment of dry cell weight and protein in shaking cultures; PDB and extract media + 10% (v/v) SPEX.

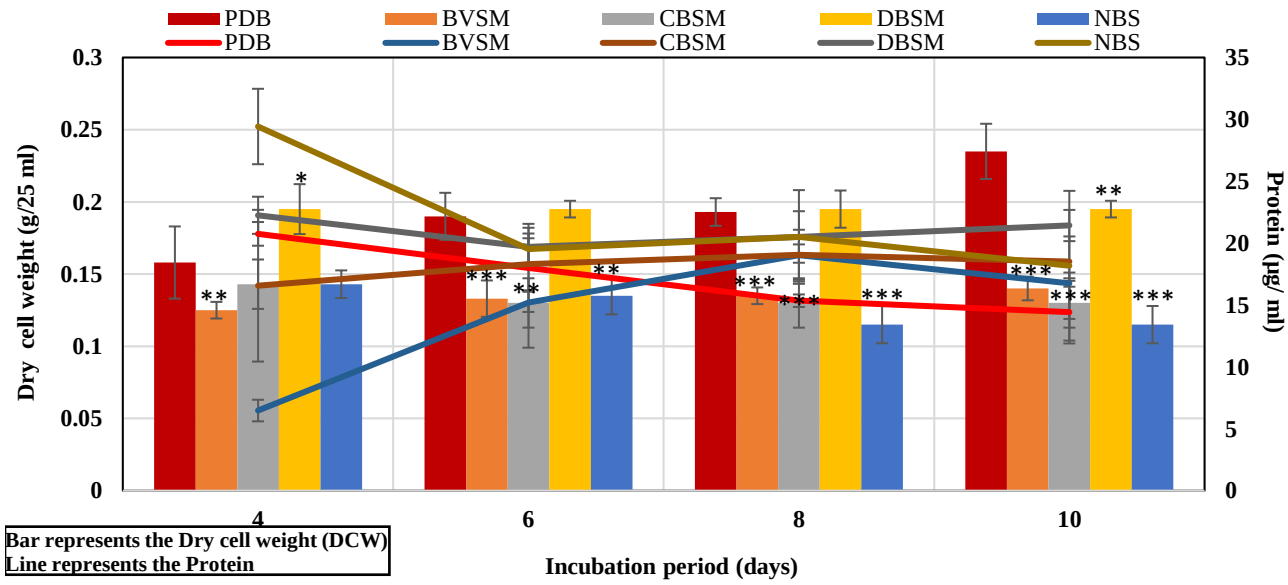

Fig. 3. Assessment of dry cell weight and protein in static cultures; PDB and extract media + 10% (v/v) SPEX.

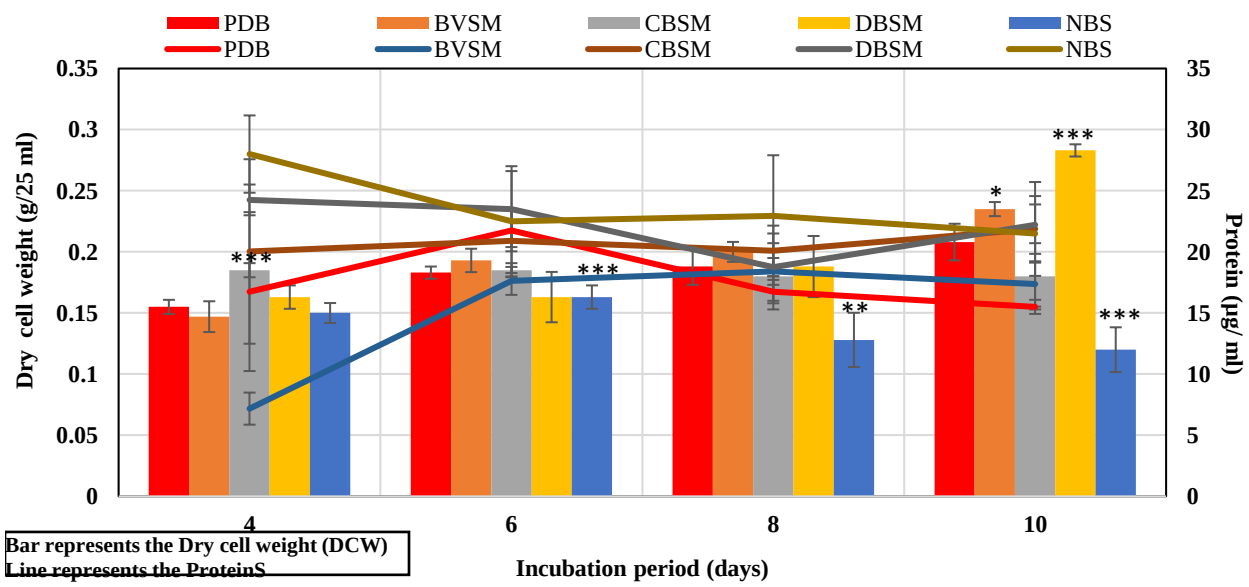

Fig. 4. Assessment of dry cell weight and protein in shaking cultures; PDB and plant-extract media + SPEX 20% (v/v).

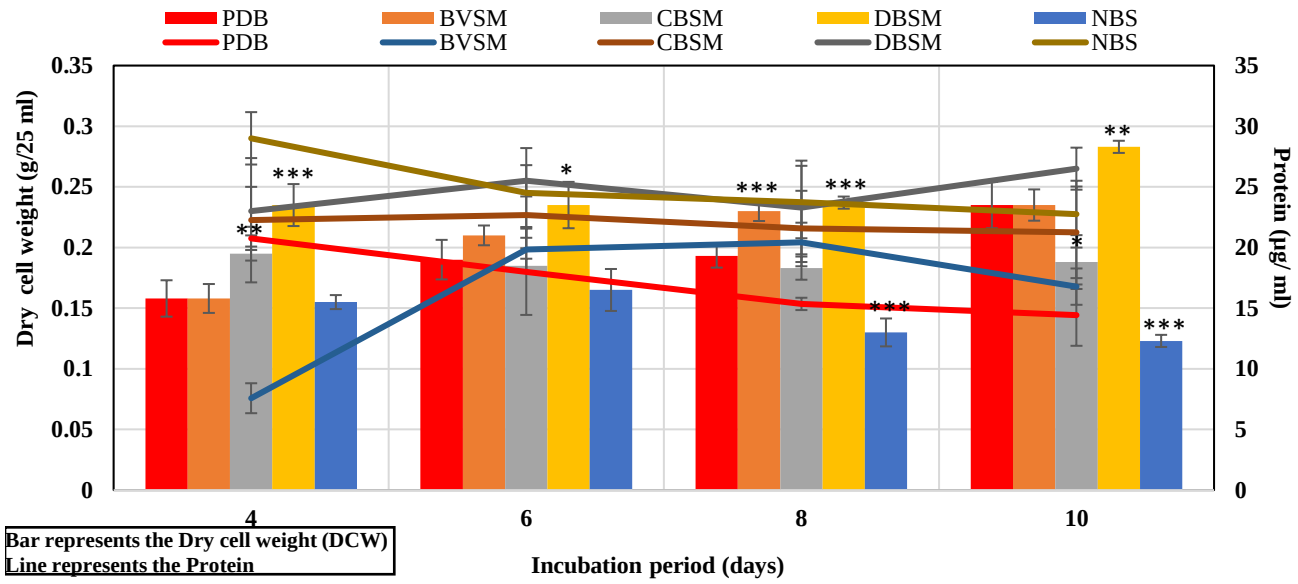

Fig. 5. Assessment of dry cell weight and protein in static cultures; PDB and plant-extract media + SPEX 20% (v/v).

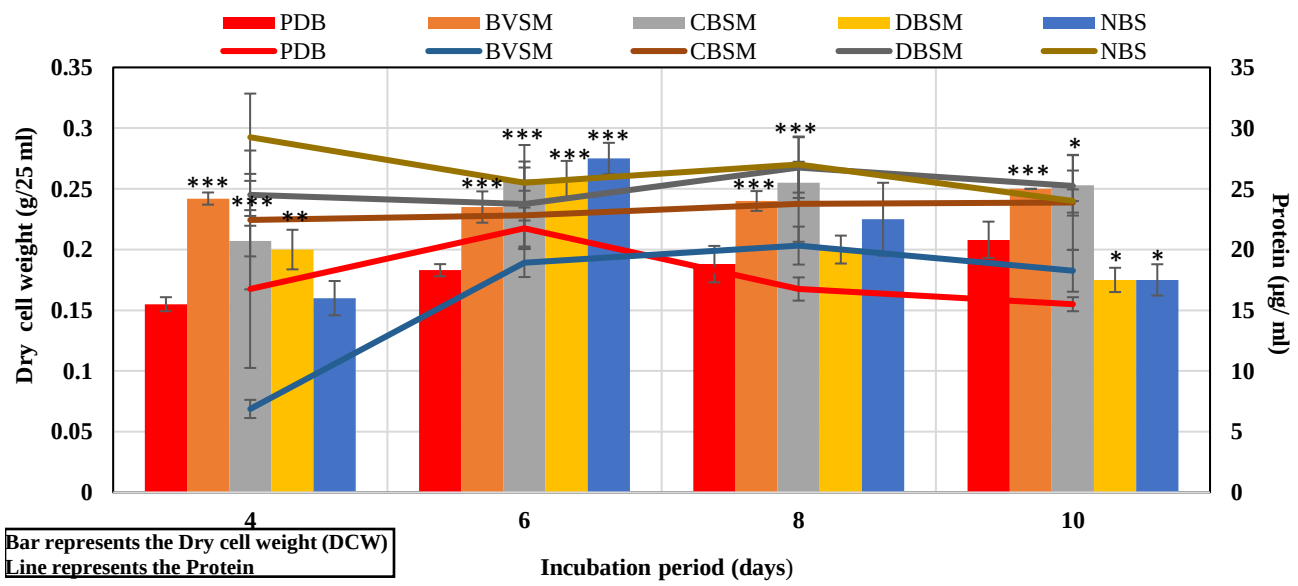

Fig. 6. Assessment of dry cell weight and protein in shaking cultures; PDB and extract media + 30% (v/v) SPEX.

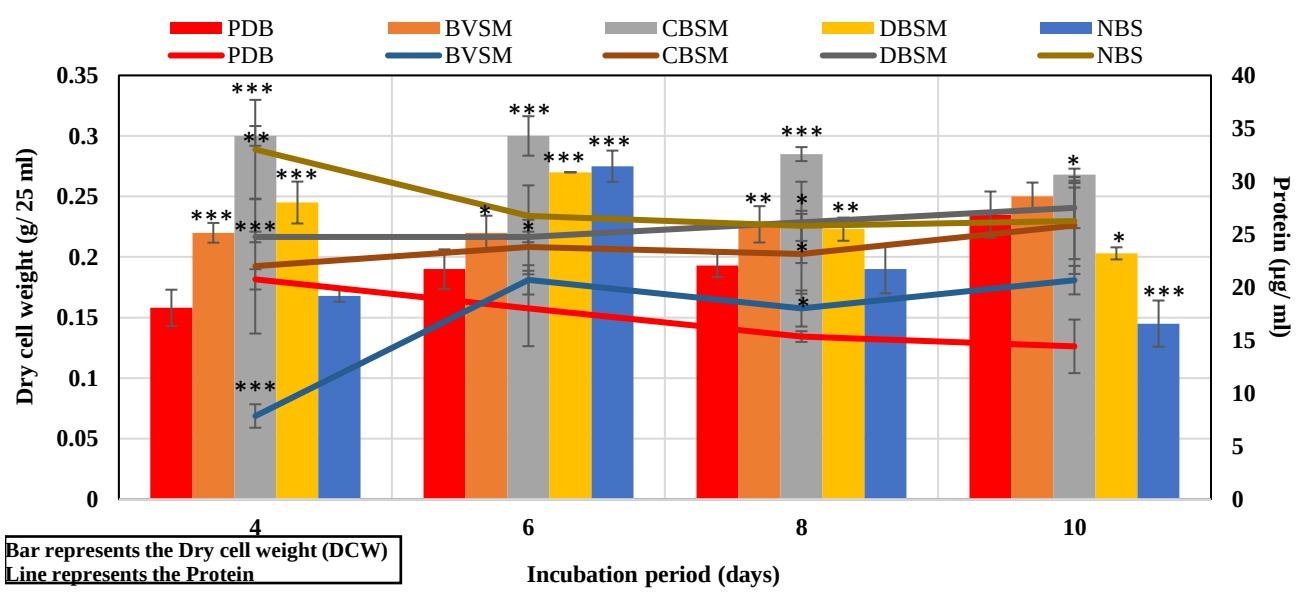

Fig. 7. Assessment of dry cell weight and protein in static cultures; PDB and extract media + 30% (v/v) SPEX.

Table 2 Estimation of the pH and sugar content in the initial concentrations of various extracts.

Extract name	Abbreviated for	% Concentration (w/v)	pH	Sugar concentration (mg/ml)
BVEX	Bougainvillea branch extract	20	6.42 ± 0.02	0.257
CBEX	Coconut bark extract	20	5.70 ± 0.02	1.048
DBEX	Date bark extract	20	6.03 ± 0.03	1.018
NBEX	Nimtree bark extract	20	6.47 ± 0.02	1.342
SPEX	Sugarcane peel extract	25	5.65 ± 0.03	6.235
SPEX	Sugarcane peel extract	35	5.60 ± 0.02	8.726

Table 3. Determination of initial reducing sugar content, pH in PDB and used extract media combinations.

S. No.	Abbreviation of the medium	Abbreviated for	Sugarcane peel extract concentration % (v/v)	Reducing sugar content (mg/ml)	pH
1.	PDB	Potato dextrose broth	------	22.5 ± 0.02	5.60 ± 0.2
2.	BVSM	Bougainvillea branch extract + Sugar cane peel extract + Mineral salt solution	10	1.50 ± 0.2	6.50 ± 0.2
			20	2.30 ± 0.2	6.32 ± 0.2
			30	3.45 ± 0.2	6.15 ± 0.2
3.	CBSM	Coconut bark extract + Sugar cane peel extract + Mineral salt solution	10	2.64 ± 0.1	6.60 ± 0.2
			20	4.80 ± 0.2	6.45 ± 0.2
			30	6.40 ± 0.22	6.10 ± 0.2
4.	DBSM	Date bark extract + Sugar cane peel extract + Mineral salt solution	10	6.80 ± 0.2	6.60 ± 0.2
			20	8.94 ± 0.2	6.50 ± 0.2
			30	10.77 ± 0.25	6.38 ± 0.2
5.	NBS	Nimtree bark extract + Sugar cane peel extract	10	2.78 ± 0.02	6.38 ± 0.2
			20	7.68 ± 0.2	6.30 ± 0.2
			30	15.864 ± 0.25	6.10 ± 0.2

Regarding the growth on extract media combinations, it seems that the BVSM had inadequate nutrients (Table 2) and despite the supplementation of SPEX as an extra carbon source (Table 3), nutritional requirement of fungus could not be fulfilled. In the case of DBSM, sugars still being present in low concentration extract media was able to support fungal growth due to presence of another nutrient sources. Moreover, the increased growth in shaking culture was due to the sufficiency of oxygen and fungal contact with nutrients. In general, the DCW production on extract media was found to be better than that of PDB. The pattern of DCW values in NBS, DBSM and CBSM; being highest on 6th day followed by growth reduction on day 8 onwards have been also presented by Ogbonna *et al*., (2015). The researchers stated that because nutrients are not additionally provided in a medium therefore, growth of *Aspergillus niger* drops progressively after reaching to its highest at day 6. Shin *et al*., (2009) also mentioned that the reduction of biomass occurs in between last growth stages because of apoptosis, cell aging or several additional factors including activity of certain enzymes such as proteases, chitinases and glucanases of *Aspergillus nidulans*. Alarid-García *et al*., (2021) further reveals that the utilization of various carbon sources thus production of biomass was found to be better when the cultures of *Aspergillus niger* (CDBB-H-175) were maintained in agitated tanks.

The pH of PDB was initially 5.6 while the extract-based media having SPEX 30, 20 plus 10 % (v/v) concentrations had a pH between 6.1 to 6.6 (Table 3). During shaking / stationary conditions pH of PDB approached to 4.09, 4.17 on 4th day which further declined to 3.5 and 3.34 respectively at 10th day. Generally, overall pH of extract-based media was acidic in range in comparison to that of PDB at varying length of incubation. The extract media: CBSM and DBSM (20 % SPEX concentration) demonstrated a pH of 3.82, 3.51 and 3.6, 3.78 respectively at stationary and shaking condition with a significant difference of the p values (p=<0.001) on 4th day. Subsequently NBS (30 % SPEX) with a pH of 2.81 and 2.85 in stationary and shaking conditions was significantly different (p=<0.001) in comparison to the pH of PDB at day 4 that elevated up to 4.26 and 4.38 at 10th day (Figs. 6 and 7).

The alteration of pH in PDB may possibly be due to the presence of initially elevated sugar levels; 22.5 mg/ml, which was effectively used by *A. niger* that resulted in release of organic acids within the medium thereby bringing decline in pH values. The extract media combination having varied SPEX concentration showed a similar pattern. Nasr *et al*., (2021) in a study revealed that during growth when the glucose is utilized as a source of carbon, it results in medium's acidification due to production of organic acids. However, sugar concentration in plant extract-based media was comparatively lesser than the sugar content of PDB, as a result of which organic acids secreted during sugar consumption were re-utilized thereby releasing CO_2 in 10 days of incubation causing the medium's pH to neutral. According to Bennet-Clark & La Touche, (1935) accretion of organic acid in the medium reflects its sugar content, but in due course of incubation while the starvation advances, acids vanish from medium thereby increasing CO_2 output, this statement is in accordance with our observation.

Table 4a. Determination of reducing sugar and alteration in pH of preffered extract media combinations in comparison to PDB under shaking conditions.

Incubation condition	Parameter	Medium	Concentration of SPEX % (v/v)	Incubation period (days)			
				4	6	8	10
Shaking	pH	PDB	-	4.1 ± 0.03	3.7 ± 0.01	3.6 ± 0.16	3.5 ± 0.06
		BVSM	10	4.8***± 0.03	4.8***± 0.14	5.8***± 0.11	7.3***± 0.49
			20	5.1*** ± 0.09	4.8*** ± 0.1	5.7*** ± 0.04	5.8***± 0.08
			30	4.2*± 0.07	5.0***±0.09	4.6***±0.16	5.6***± 0.38
		CBSM	10	4.0*** ± 0.04	4.6*** ± 0.17	5.4*** ± 0.09	5.4*** ± 0.10
			20	3.6***± 0.04	4.3***± 0.05	4.8***± 0.08	5.1***± 0.09
			30	4.2± 0.07	4.9***± 0.23	5.0***± 0.02	4.4***± 0.13
		DBSM	10	7.1*** ± 0.71	5.1*** ± 0.05	6.3*** ± 0.17	6.9*** ± 0.45
			20	3.8** ± 0.12	4.5*** ± 0.17	4.7*** ± 0.16	5.6*** ± 0.26
			30	3.7***± 0.06	3.8±0.04	3.9**±0.03	3.8**±0.10
		NBS	10	4.3 ± 0.22	6.6*** ± 0.07	6.8*** ± 0.04	6.1*** ± 0.04
			20	4.3*** ± 0.04	4.5*** ± 0.01	4.4*** ± 0.07	4.4*** ± 0.04
			30	2.9***± 0.05	4.1** ±0.05	4.3**± 0.33	4.4*± 0.06
	Reducing sugars (mg/ml)	PDB	-	5.5 ± 0.1	3.5 ± 0.1	3.7 ± 0.05	5.8 ± 0.03
			10	0.5*** ± 0.03	0	0	0
		BVSM	20	2.5**± 0.04	1.24**±0.05	1.1***±0.03	0.08***± 0.001
			30	2.08***± 0.01	0.2***± 0.017	0	0
		CBSM	10	0	0	0	0
			20	2.3***±0.03	0.15***±0.01	0	0
			30	1.04***± 0.02	1.3**± 0.04	0.1***±0.01	0.3***±0.01
		DBSM	10	1.3*** ± 0.38	2.2* ± 0.23	0.92***± 0.81	0.22*** ± 0.2
			20	4.9±0.05	4.9** ± 0.05	3.6± 0.06	3.0**± 0.27
			30	2.2 ***± 0.01	2.2*± 0.01	6.4***± 0.05	6.5± 0.0
		NBS	10	0.4*** ± 0.09	0.35*± 0.13	0.5***± 0.09	0.7*** ± 0.08
			20	4.31± 0.06	3.33± 0.04	2.95*±0.03	3.11***±0.04
			30	3.9*± 0.02	3.8±0.04	3.8±0.04	3.5***±0.03

Criteria for determining the level of significance: ≤0.05 = *, ≤0.01 =**, ≤0.001 = ***

Table 4b. Determination of reducing sugar and alteration in pH of preferred extract media combinations in comparison to PDB under stationary conditions.

Incubation condition	Parameter	Name of medium	Concentration of SPEX [% (v/v)]	Incubation period (Days)			
				4	6	8	10
Static	pH	PDB	-	4.2 ± 0.01	3.6 ± 0.12	3.4 ± 0.07	3.3± 0.157
		BVSM	10	4.9***± 0.09	5.8***±0.12	6.465***±0.07	6.875***±0.12
			20	4.7**±0.19	5.3±0.15	5.6±0.16	5.8±0.19
			30	3.7***± 0.07	4.1*** ±0.08	4.9***± 0.04	5.2***±0.24
		CBSM	10	3.6***±0.12	4.1***± 0.12	4.7***± 0.08	4.9***±0.11
			20	3.8**±0.19	4.4***±0.24	4.9***±0.05	5.3***±0.1
			30	3.7***±0.07	4.0***±0.07	4.4***±0.11	4.7***±0.06
		DBSM	10	5.7***± 0.09	5.1***±0.09	5.6***± 0.29	6.9***± 0.04
			20	3.5***±0.13	4.5***±0.04	4.6***±0.16	5.0***3±0.17
			30	4.2±0.05	4.5***±0.22	4.8***±0.18	5.3***±0.19
		NBS	10	3.9*± 0.13	6.8***± 0.13	7.0***±0.078	6.8***±0.06
			20	4.5***±0.06	4.3***±0.01	4.0***5±0.05	4.5*** ±0.01
			30	2.8***±0.03	4.***1±0.02	4.1***±0.02	4.3***±0.03
	Reducing sugars (mg/ml)	PDB	-	4.5± 0.1	3.8 ± 0.04	3.9± 0.03	4.5 ± 0.03
			10	0.45***± 01	0	0	0
		BVSM	20	2.7**± 0.02	0.4***±0.05	0.3***±0.003	0.2***± 0.001
			30	2.1** ± 0.01	0.2***± 0.02	0	0
		CBSM	10	0.42***± 0.01	0	0	0
			20	2.3**± 0.03	0	0	0
			30	4.8±0.04	2.6***±0.02	2.5***±0.02	1.9*** ± 0.03
		DBSM	10	0.33***±0.04	1.9±0.07	0	0
			20	3.17**±0.02	0.9***±0.01	0.24***±.002	0.1***±0.07
			30	2.1**±0.03	2.1***±0.04	4.3±0.09	4.1±0.6
		NBS	10	0.5***± 0.2	0.4***±0.01	0.4***± 0.01	0.3***±0.01
			20	3.15*±0.04	3.1±0.04	2.96**±0.03	2.59***± 0.02
			30	5.0±0.1	1.9***± 0.03	2.7*±0.08	3.21*± 0.07

Criteria for determining the level of significance: ≤0.05 = *, ≤0.01 =**, ≤0.001 = ***

Table 5. Citric acid concentration in PDB/ NBEX with varying SPEX concentrations (day 4).

S. No.	Medium	SPEX Concentration % (v/v)	Citric acid content (g/l)	
			Shaking cultures	Static cultures
1.	PDB	------	27	24
2.	NBEX+SPEX	10	3.91	3.83
3.	NBEX+SPEX	20	6.0	5.77
4.	NBEX+SPEX	30	7.33	7.28

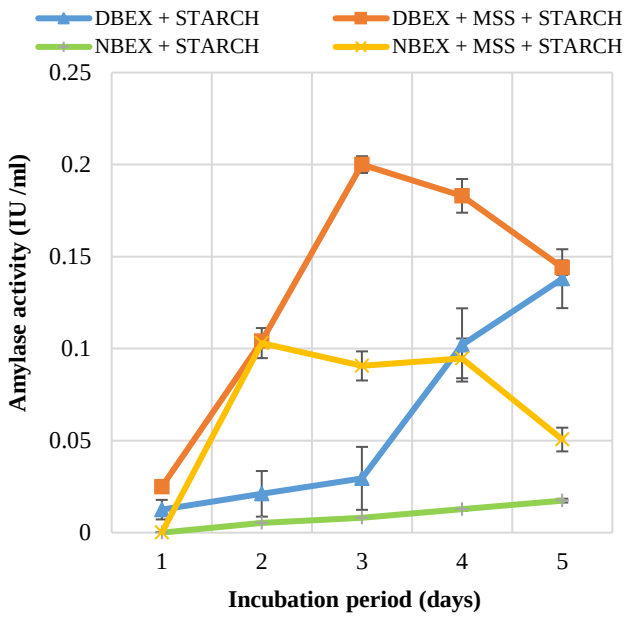

Fig. 8. Quantification of amylase concentration in DBEX/ NBEX +combinations.

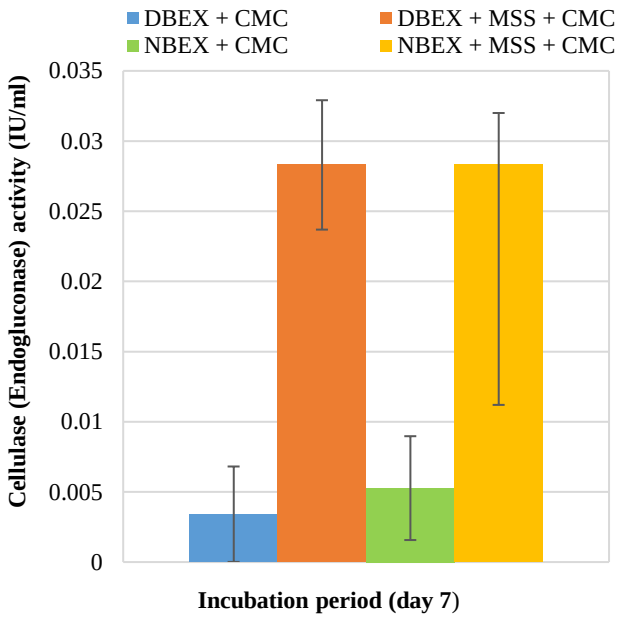

Fig. 9. Quantification of cellulase (endogluconase) concentration in DBEX/NBEX + combinations.

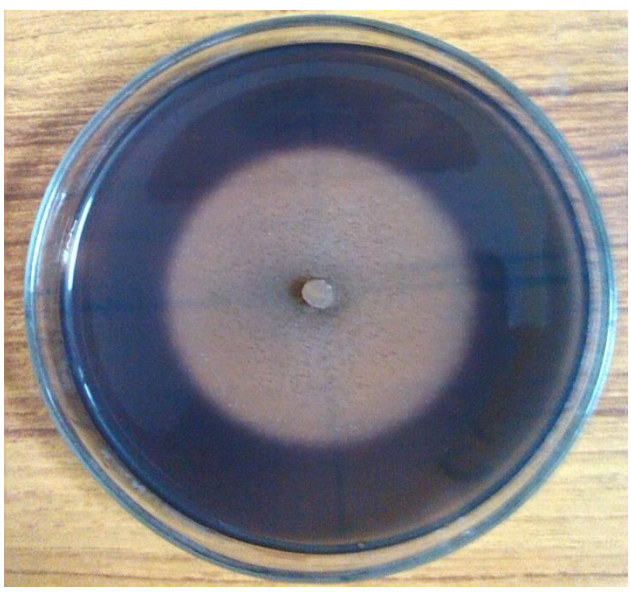

Fig. 10. Plate screening assay for amylase production.

Fig. 11. Plate screening assay for cellulase (endogluconase) production.

The un-inoculated media namely NBS, DBSM. CBSM and BVSM, having 10% SPEX concentration had an initial concentration of sugar as 2.78, 6.8, 2.64, 1.5 mg/ml respectively (Table 3). The sugar levels are considerably low in comparison to the reducing sugar concentration (22.5 mg/ml) of PDB. During shaking conditions excluding CBSM, reducing sugars on day 4 were detectable in other extract media combinations but became un-detectable as growth approached to 10th day nonetheless, NBS contained detectably low concentrations of sugars during the whole period of incubation (Table 4a). A detectable concentration of reducing sugar was found in PDB not only during shaking but static conditions as well, when incubation period reached day10 (Table 4a).

The un-inoculated extract media combination with 20/30 % SPEX specifically NBS, DBSM, CBSM and BVSM contained an initial reducing sugar levels of 7.68, 8.94, 4.8, 2.3 and 15.864, 10.77, 6.40, 3.45 mg/ml correspondingly (Table 3). These values suggest that the un-inoculated extract media combinations with greater SPEX concentrations had a greater reducing sugar content as that of the media having SPEX 10% v/v concentration, hence substratum was competently used during active growth for biomass production in both shaking or static conditions (Table 4a and 4b). Peksel & Kubicek, (2003) while observing effect of sucrose concentration on the production of citric acid stated that an osmotic stress developed nearby the surroundings of a fungus is due to raised sugar levels that is controlled by tyrosine kinase (an internally released protein). It also acts as an osmo-sensor which is influenced by osmotic variation and mediates ionic flux pathways thus maintaining the solute balance beyond the cellular membrane of fungi. Meanwhile a direct link between the biomass production/ citric acid accumulation and initial sugar content existed. Therefore, increased sucrose levels in the medium resulted in higher sugar consumption thereby increasing biomass production whereas lesser biomass production was observed in a medium with reduced sugar levels. The results of our study corelates with this fact nevertheless, the near progression towards last stages of growth or as starvation begins, limited carbon stocks occurring in fungal cells as biomass, or other forms must have liberated into the extract media. This typical phenomenon resulted in the detection of measurable reducing sugar concentration thereby lowering biomass, which is also explained in two separate studies performed by Paulillo *et al*., (2003) and Shin *et al*., (2009).

The protein content on 8^{th} and 6^{th} day during shaking/stationary conditions in extract-based media having SPEX 10 % (v/v) concentration was almost at par as that of PDB. A significant (p=<0.001) and consistently decreased protein value was found during shaking and static conditions in BVSM medium with varying SPEX concentrations. However, in NBS with varying SPEX concentrations, significantly increased protein values were observed during static and shaking conditions at 4^{th} day (Fig. 2-7). The protein content in shaking plus static cultures at 4^{th} day in CBSM with varying SPEX concentrations was comparable, whereas in DBSM with varying SPEX concentration, values were not only comparable to that of PDB but also increased significantly (p=<0.001). Protein concentration in DBSM and CBSM + SPEX 10 % (v/v) was significantly raised in static and shaking cultures on 10^{th} day (Fig. 2-3). The protein content in NBS and BVSM during static and shaking conditions was equivalent of the protein content of conventional medium. The extract media NBS, DBSM, CBSM and BVSM + SPEX 30 % (v/v) and NBS, DBSM and CBSM + SPEX 20 % (v/v) under static and stationary conditions on 10^{th} day showed significant increase in protein content. The proteins, during several fungal growth stages are secreted extracellularly mostly comprising of the enzymes having industrial and environmental significance, required to degrade the complex polymers existing in nature. In this regard, Wösten *et al*., (1991) revealed that the cell wall of a fungus acting as barrier may not permit the entry of larger molecules so the proteins must come across the cell wall pores in order to be secreted into the medium. That is why the enzymes bearing environmental and industrial significance are mostly extracellular in nature. The report also mentioned that certain enzymes are released during idiophase in large concentrations. Idiophase is a growth phase when the fungal propagation halts and production of biomass ceases. These findings are in accordance with our results and suggest that accumulation of the protein based metabolic products, during end stages of the growth contributed to elevated protein content of a medium.

Aspergillus niger was screened for enzyme production specifically endoglucanse, amylase and laccase. The fungus produced halos; clearing of DBEX medium where starch was hydrolyzed by amylase during the exposure of plates to iodine vapours (Fig. 10). Whereas hardly visible clearing zones (halos) were developed in the medium where cellulase hydrolyzed CMC (Fig. 11), after treating the plates as mentioned by Kasana *et al*., (2008) with Gram's iodine solution. Nevertheless, no clearly visible zones were produced by *Aspergillus niger* in DBEX medium supplemented with the dye bromophenol, showing absence of laccase activity by this fungal specie. Quantification of enzyme (amylase/cellulase; endogluconase) production on preferred extract media was performed by using submerged fermentation (SmF) technique. For amylase estimation, extract-media containing starch substrate was incubated under shaking condition for 5 days followed by enzyme assay within each 24 hours. The results show that the amylase production was low in NBEX and DBEX media alone (Fig. 8) nonetheless, same extract media combination with MSS produced better enzyme levels. The highest enzyme activity, 0.1 IU/ml was found on 2^{nd} and 3^{rd} day in NBEX +MSS + starch containing media whereas, DBEX +MSS + starch amendment showed values up to 0.2 IU/ml with a subsequent drop in enzyme levels in later days. In the case of cellulase (endogluconase) production assays, the results were observed after 7 days of incubation under shaking conditions. Enzyme levels in NBEX excluding MSS were better as compared with DBEX alone (0.005 and 0.003 IU/ml respectively). However, enzyme production in the same extract media improved when amended with MSS. Mahmood *et al*., (2021) and Shahid & Nadeem (2015) in their study stated that in order to produce highest growth or enzyme concentration, a suitable culture medium must be used. The enzyme producing potential of *A. niger* was therefore, tested on the chosen media (DBEX and NBEX) which showed most promising growth assay results earlier. These media were used alone or in supplementation with MSS along with the respective substrates. The low amylase

production in DBEX and NBEX amended only with starch (Fig. 8) indicates that enzyme activity depends on the presence of various inorganic constituents. Generally, the media formulations utilized for enzyme production contain mineral salts therefore, extract media + MSS produced enhanced enzyme levels on day 2-3 followed by the drop in values on day 5. A similar pattern was observed for the production of cellulase (endogluconase) in these extract media on day 7. The decline in enzyme values in later days of incubation may probably be because of the toxic waste accumulation or nutrient scarcity as suggested by Adegbanke *et al*., (2021). Similarly, Ogbonna *et al*., (2015) in their report demonstrate that enzyme levels in a fermentation medium may drop due to its high viscosity which interfere with the uptake of oxygen by fungal cells causing delayed metabolism, enzyme production and cell division.

In a separate study Mohapatra *et al*., (2018) observed that shaking cultures containing substrate levels up to 1 percent, resulted in higher cellulase concentration; 0.149 U/ml during incubation period of 6 days which declined as the incubation period extended. Our study corresponds to these findings that enzyme production in the medium occurred in presence of CMC levels of 1% weight by volume, during incubation period of 7 days (Fig. 9). Nonetheless, the effect of other physiological factors on enzyme production may also be taken into consideration. In similar context Haq *et al*., (2021) while studying the temperature effect on production of β-galactosidase by *Aspergillus oryzae*, mentioned that the best enzyme activity was noted at 30°C in an incubation time of 5 days (120 h) which was ultimately affected by either increasing temperature or incubation time. The results of our study also indicate that when the cultures were kept at temperature 30±2°C the maximum enzyme activity was observed in 5- or 7-day incubation time after which decline in enzyme levels was clearly noted.

Citric acid production by the fungus during shaking and stationary condition was assessed then compared to that in PDB on NBEX media with varying SPEX concentrations of 30, 20, 10 % (v/v). It was observed that stationary and static cultures of PDB had many folds higher concentration of citric acid as compared to the citric acid values in media having varied SPEX concentrations (Table 5.). The medium NBEX with 10% v/v SPEX showed low concentration of citric acid in contrast to NBEX with 30% (v/v) SPEX in which higher values were attained. The cultures maintained at shaking conditions (excluding NBEX + SPEX 10 % (v/v) produced more citric acid in comparison to the cultures kept at static condition.

The production of organic acids at increased concentration is generally linked with higher sugar content of a medium (Table 3.) as explained by Behera *et al*., (2021) that for strains of *A. niger*, 10-14% of the initial sugar concentration was required for citric acid production. However, no acid production was observed at <2.5% sugar concentration. Our results also correlate with these findings and indicated that low sugar content in varying combinations of extract media irrespective of shaking or static conditions also produced low acid levels. Apart from sugar concentration, other factors such as pH also influence citric acid production. Studies concerning with the variation of pH during acid production by *A. niger*, evidently showed that citric acid production began at a pH of 3.0 and < 2.0 being an optimum pH (Magnuson & Lasure, 2004). In another study Chergui *et al*., (2021) demonstrates that initially a pH of >5 is required for spore germination followed by a successful citric acid production, the pH of a medium would fall naturally reaching below ≤2. Similar pattern was noticed in extract medium NBEX + 30% (v/v) SPEX where the pH value dropped at 3.0 in static and shaking conditions. Interestingly the presence of organic acid was noted in PDB at a pH of >4.0 and media combinations; NBEX + SPEX 10, 20 % (v/v). Our results corroborate the findings of Andersen *et al*., (2009) according to whom the production of acid was greater at pH 4.5 pH and lower at pH 2.5 or 6.0.

Conclusion

Most of the plant-based extracts have been assessed for the antimicrobial capability. However, the novel application of these extracts as the main ingredient in the development of culture media for fungal growth explores a new horizon in the field of Microbiology. The laboratory formulated extract media combinations were found to be at par or even better when compared with the reference medium PDB. In this regard CBSM, DBSM and NBS used along with varying concentrations of SPEX produced reproducible results for fungal DCW, extracellular protein, reducing sugars and alteration in pH of the media. The extract combinations also showed better results for citric acid, amylase and endogluconase production. Therefore, it can be concluded from the above results that the extract media combinations have been found to be suitable for growth and other activities of *Aspergillus niger*.

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