Production of Cellulase with Potential Use in Sugarcane Bagasse Saccharification by Fungi

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Abstract

The research focused on cellulase synthesis with possible application in sugarcane bagasse fungal saccharification. Under this regard, the goal is to examine the operating parameters (temperature and humidity) in which cellulases of the Penicillium sp., Rhizomucor sp. fungus are produced. INCQS 40331 (CFAM 422) and Trichoderma koningii, which use as natural sugarcane bagasse (NSB) and pretreated by acid-alkaline solution (AAB) and hydrogen peroxide (HPB), respectively. The research was conducted using three-tier factory design (32) with central points. The findings indicate that the Trichoderma koningii (8.2 IU/g substratum), followed by Penicillum sp (1.7 IU/g substratum), was the most appropriate cellulase fungus. In addition, the best results have been achieved with NSB for all fungi. Statistical analysis revealed that the temperature has a larger impact on cellulase production by the fungus assessed.

Keywords: *Fungi, Enzyme production, Sugarcane bagasse, Glycerol, Enzymatic Saccharification.*

1. INTRODUCTION

Fungus, in particular cellulolytic fungi, are regarded as significant degrading agents of paper documents. Certain filamentous fungus often colonise paper and may destroy cellulose fibres via cellulolytic enzymes or change aesthetic and visual appeals through the release of weak acids or colours.

Native wood cellulose is utilised for paper manufacturing. Paper may include hemicelluloses (wood polyoses), lignin and additives, such as fillers and colours as well as cellulose fibres. Filamentous fungi often linked with paper degradation may, by synergistic activities, dissolve cellulose fibre (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91), and β- glucosidase (EC 3.2.1.21) resulting in severe cultural and historical harm to paper materials (Reese and Downing 1951; Nyuksha 1983; Ciferri et al. 2000). Porck (2000) observed that the cellulose molecules' hydrolytic breakdown is the most common response, in particular when the presence of water in the storage environment plays a key role. Microbial activity is, in fact, mainly dependent on the paper's water activity (aw). It is a free water measurement that is used to develop the mould and is defined as the vapour pressure ratio of the substratum water and the vapour pressure of pure water at the same temperature and relative humidity (RH). The hydrolytic process is governed by the combined effects of aw, the temperature, pH and relative humidity according to the standard reaction. The moisture contents of a paper can reach between 8 and 10% at an HF level higher than 65% and a temperature higher than 20°C, with consequent water activity (aw) higher than 0.65, with the microbial spores germinating and developing by using paper as a growth substratum, affecting the cultural value object. Prieto and Silva (2005) have found that the severity of microbial contamination is affected by the atmospheric weather conditions.

2. LITERATURE REVIEW

Hongkun Li et. al. (2021) A percentage of plant lignin and cellulose may be degraded by endophytic fungus within the plant. Endophytic Penicillium is one of the industrial micro-organisms that produces enzymes that may be released into the extracellular space with a full enzyme system. Ancient tree species' natural development from unique natural regions into cellulase-producing strains with outstanding features offers a potential path for future industrial enzymes. A new fungal endophyte, Penicillium oxalicum R4, has been effectively isolated and tested with a greater Taxus cuspidata cellulase activity. Under optimised cultivation conditions achieved by a Box–Behnken design (BBD) and an artificial Neural Network–Genetic algorithm

(ANN–GA), P. oxalicum R4 production yields (1.45, 5.27, and 6.35 U/mL), about 1.60 times, 1.59-times, and 2.16 times higher than non-optimized crops, were of Filter Paperase(F Pase), Carboxymethyl Cellulase(CMCase) and β-glucosidase (βGLase), respectively. The finding of endophytic fungal cellulase strains in unique natural geographical habitat, such as Taxus cuspidata, known as live plant fossil, offers fresh study guidelines on future industrial enzymes.

Gilson Araújo de Freitas et. al. (2019) The selection of microbes with the most composer potential allows for efficiency improvement and time reduction. The isolation of fungi is particularly damaged since throughout the composting process they are the primary contributors for the breakdown of components of complex decomposition. This study examines fungi having cellulolytic enzyme potential, the isolation and screening techniques of these fungi, and the methods for assessing possible enzyme tests. The fungi which break down rich cellulose residues are mostly decomposers of ruminal residues and contain a variety of enzymes with the degradation capability of complex organic molecules, such as cellulose, hemicellulose, aromatic acids and some proteins. Therefore, specialised glasses to convert this kind of waste may be created by indicating fungus that enhance composting efficiency.

Mohamed M. Ahmed et. al. (2018) Fungi are well-known decomposing agents in ordinary organic matter and in particular of cellulose material, and are thus regarded to be the principal cellulose generating microbe. The objective of this research was to identify and test the capacity of cellulolytic fungal strains to generate cellulolytic enzymes from desert soils that are under environmental stress. Fourty-three fungal strains were isolated from various locations in Aswan University campus in addition to two kinds and were able to breakdown cellulose with varied dimensions. High, moderate and low cellulolytic activity were classified as fungal species. Carboxymethyl-cellulose "CMase" (endoglucanase) testing was performed on cellulase activity. Fusarium dimerum and Rhizopus oryzae were the highest isolate cellulase production. At 9th, 11th and age C, the ideal conditions for fusarium dimerum and average cellulase activity are pH 5, 35 Rhizopus oryzae respectively.

Sriyamjalla Santoshkumar et. al. (2015) The research focused on isolation and screening, optimization, and stability investigations of thermophilic cellulase producers for maximal cellulase output. Telangana was isolated from 15 thermo-film cellulas generating fungus (vegetable market compost, mushroom compost, horse dung, municipal waste, bird nests, decomposing trash, furnace soils, cow dung, zoo dump, industrial waste). All fungal isolates have been tested for cellulase production. The greatest cellulase activity in screening was found in Scytalidium thermophilum, SKESMBKU02, and chosen for further research. The findings revealed S. High cellulolytic activity at 45°C and pH 5.0 - 6.0 was discovered to be thermophilic SKESMBKU02. Enzyme production optimization was investigated in various sources of carbon and nitrogen. The activity in endo and exoglucanase were greater in glucose-containing medium than their carbon source and xylose. Yeast extract and peptone were respectively excellent sources of nitrogen for endoglucanase and exoglucanase activity. In (NH4)2SO4 and NH4Cl, the organism exhibited maximum dry weight. The S generated exo and endocellulases. SKESMBKU02 thermophilic was very stable at pH 8.0 and the temperature was 75 °C. The findings show that the fungus generated endo and exocellulases were more stable at high temperatures and alkaline pH.

Sujatha E, Santoshkuar S and Shiva Krishna P (2014) Fifteen fungal isolates of Andhra Pradesh were insulated from various thermal environments (vegetable market compost, pillows, horse dung, municipal trash, birds nests, decaying litter, furnace soils, animal dung, zoo dump and industrial waste). The capacity of all fungal isolates to generate cellulases was tested. The findings revealed that Skesmbkul01 had strong cellulolytic activity at 45°C and pH–5.0. The optimization of enzyme production was investigated at 1 and 0.2 percent respectively in various carbon and nitrogen sources. In media containing glucose the activity of endo- and exoglucanase is greater, followed by xylose and lactose. KNO3 is an excellent endoglucanase and urea nitrogen supply for exoglucanase operations. There was maximum dry weight in malt and peptone extract. S.thermophilum culture filtrate was dialyzed over night against distilled water and was utilised as the enzyme source. The SKESMBKU01 exo and endocellulases are very stability at pH 8.0 at a temperature of 85°C. The findings suggest that SKESMBKU01 endo and exocellulase are more stable at high temperatures and alkaline pH.

3. MATERIALS AND METHODS

Isolation, maintenance and identification of fungi

Sugar cane piles, corn and sugarcane bagasserie, fruit, wood, and animal feed have been isolated in the north-western part of S~ao Paulo State: S~ao Jose do Rio Preto Municipalities (49°220 45″ W – 20°490 11″S), Potirendaba municipalities (49°220 38″ -21°020 34″s) and Colina municipalities (47°320″2 27″W – 20°420 48‴S), Brazil. Samples (approx. 0 5 g) of sterile nutrient medium (pH adjusted to 5 0) composed of (g l1) were collected and transferred directly to Erlenmeyer's 50 0 ml flasks, containing (n 10 0): glucose (10 0), (NH4)2SO4 (1 4), KH2PO4 (2 0), CaCl2 (0 3), MgS O47H2O (0 2), peptone (5 0), yeast extract (2 0), urea (0 3), two-squamous (0 2gl1 each) and 1 ml 1 of the trace e. (FeSO4 7H2O 5 0 mg ml1 ; MnSO4 H2O 1 6 mg ml1 ; ZnSO4 7H2O 1 4 mg ml1 ; CoCl2 2 0 mg ml1). Flasks have been incubated for 24 hours at 45°C, below 100 rev min1. Then, 0 1 ml of this preculture was spread aseptically on Sabouraud plates, incubated at a temperature of 45°C, for 24-72 hours. Repeated streaks of fungal colonies with distinct morphological features have been identified. On Sabouraud agar slants, pure crops were grown for future storage and identification. Stock crops were kept at 80°C, under the solution of glycerol (20% v/v).

The colony shape studied under a stereomicroscope preliminarily identified all fungal strains (Leica EZ4; Wetzlar, Germany). Moreover, the morphology of the tiny reproductive structures was examined using cotton-blue stained mounts. Such morphological features have been utilised to identify all genus-level isolates. The chosen strain (JCP 1-4) was also identified using molecular markers after morphotyping. The primary pair ITS4 and ITS5 for the internal ribosomal DNA spacer region was sequenced (White et al. 1990). The methods reported in Arcuri et al followed the PCR conditions, DNA quantification and sequencing (2014).

Forward and reverse sequences in contiguous BIOEDIT v 7 1 3 were constructed (Hall 1999). Contigs were utilised in the NCBI-GenBank database to query homologous sequences. We obtained reference strains sequences, based mostly on the research of Brink et al. (2012) and Walther et al. from the CBS-KNAW Fungal Biodiversity Centre (2013). The line was constructed in MAFFT v. 7158. (Katoh and Standley 2013). The phylogenetic tree was inferred using MEGA v 5 05 (Tamura et al. 2011) using the adjoining Kimura 2-parameter method as replacement model and deficiencies were removed. We computed 1000 pseudo-replicates for branch support

Screening for Cellulase-Producing Fungi

Each fungal isolate was cultivated on a medium of cellulose with red congo for 4 days at a temperature of 30 ± 2℃. All Petri plates with Congo red solution was put in (1% w/v), at room temperature for 15 min, and then the solution was poured out before the solution was added to 1 mol/L NaCl for a while.

Preparation and analysis of substrates

Destilaria Itaúnas S/A (DISA, Espírito Santo, Brazil) provided Sugarcane bagasse. The material was dried at a constant weight at 55 °C and processed in a laboratory mill to an average of 6 to 20 mesh (2.36–0.85 mm). The following substance is called natural bagasse.

Acid-alkaline solution pretreated bagasse: 10 g bagasse were suspended in 200 mL of a 1% (v/v) sulphuric acid solution. The suspension was removed for about 40 minutes at 100°C; the pre-treated bagasse was filtered and washed with hot water until it reached neutral pH. 200 mL of a 7% sodium hydroxide (w/v) solution was then added to the washed acid-pretreated bagasse, and the suspension was stored at 100°C for about 40 minutes. Afterwards, the pretreated bagasse rinsed by neutral pH and 50 mM pH citrate buffer with hot water. The solid residue was dried at a temperature of 55 °C to a consistent weight.

Bagasse was also processed with a hydrogen peroxide solution, as follows: 15 g of bagasse was suspended in the pH 11.5 solution of 7.3% (v/v) of 600 mL of hydrogen peroxide. The suspension was agitated at a temperature of 25°C for 60 minutes and the pretreating solid was filtered and washed until the neutral pH was achieved. These methods have been modified by Guilherme et al (2015).

Chemical analyses were conducted on all substrates (wild bagasse and pre-treated acid-alkaline bagasse and peroxide hydrogen solutions). The content of ash and humidity was carried out in accordance with the methodology provided by Morais et al (2010). In a Perkin Elmer CHNS/O 2400 Analyzer the hemicelulose and cellulose content was quantified by the Protocol proposed by Marais et al., for the analysis of elementar composition, corresponding to the percentage content of mass of the elements carbon (percent C), hydrogen (percent H), sulphur (percent S), nitrogen (percent N), and oxygen (O percent) (2010). Non-structural extractive

agents, such as fatty acids, alcohols of long chain, waxes, resins, phenolic compounds, terps, essential oils, sugar, salts, etc. (Rojas et al., 2014) were extracted as solvents in Sohlet devices using toluene/ethanol (1:1). Morais et al. modified the technique (2010). The content of Klason lignin was measured by conventional TAPPI T222 OM-11 technique (Technical Association of the Pulp and Paper Industry, 2011).

Enzymatic production

Enzyme (IU/gsubstrate) activity derived from the SSF of T. Penicillium sp. Koningii Rhizomucor and sp. Table 1 is given. Temperature and humidity values (50 percent, 60 percent and 70 percent), utilising natural sugarcane bagasse (NSB) and bagasses pretreated with acid-alkaline solution (AAB) and with hydrogen peroxide (HPB). In a review conducted by Paramjeet et al. (2018), the culture temperature may be seen between 25°C and 45°C with the fungal strain.

Table 1

Endoglucanase activity of the crude broth after 72 h of SSF of different fungi using sugarcane bagasse as substrates, as follows: natural (NSB), acid-alkaline pre- treatment (AAB) and hydrogen peroxide pretreatment (HPB).

Run	Variables		*Trichoderma koningii*			*Rhizomucor* sp.			*Penicillium* sp.		
	T (°C)	MC (%)	NSB	AAB	HPB	NSB	AAB	HPB	NSB	AAB	HPB
1	28	50	8.20	1.23	3.39	1.22	0.81	0.47	0.04	0.04	0.01
2	33	50	7.43*	0.86	1.50	0.26	0.08*	0.25	0.06	0.04	0.02
3	38	50	2.24	0.33	2.10	0.14	0.02	0.11	0.06	0.08	0.07
4	28	60	6.05	0.65*	3.19*	1.69	0.39	0.50	0.09	0.02	0.06
5	33	60	3.48	0.82	0.79	0.77	0.64	0.56	0.13	0.02	0.02
6	38	60	2.62	0.73	0.03	0.20	0.02	0.02	0.12	0.05	0.08
7	28	70	5.71	1.28	1.08	1.71	0.39	0.62	0.10	0.03	0.05
8	33	70	2.21	0.52	1.10	0.82	0.82	0.40	0.41	0.02	0.03
9	38	70	2.51	0.32	0.12	0.28	0.05	0.12	0.09	0.15	0.13
10	33	60	3.45	0.88	0.92	0.67	0.70	0.55	0.25	0.00	0.02
11	33	60	3.87	0.88	0.77	1.16	0.81	0.53	0.17	0.00	0.02

* outlier.

Determination of Cellulases Activity

Filter paper activity was described by Hankin as filtering paper activity (Fpase), estimated at 100 mM by using Whatman No. 1 (FP, 50 mg, 1.0 cm to 6.0 cm), pH 5.0 by citrate buffer 1.0 ml, containing 0.5 ml of crude enzyme solution (DCES) suitably diluted for 60 min at 50 c., while DNS was used to examine the released reduction of the sugar yield. The activity of one filter paper unit (FPU) was defined as the volume of 1 ml of crude enzyme released per minute and 1 ml of glucose.

In Miller, activity of carboxymethyl cellulase has been described. Each strain in 150 mL conical flask containing 50 mL of basal medium, modified with HWS (10g/L) as the only source of carbon was grown to evaluate the capacity of fungal isolates to generate cellulose. Briefly, 0.2 mL of crude solution and 1.8 mL of 1 percent CMC in a 100 mM sodium phosphate buffer at pH 5.5 were added in the Reaction System, 50 daC in a bath for around 25 min. Afterwards, the reaction system was halted by the addition of DNS reagent and placed at 100 − C for 15 min in a water bath. The OD was measured at 520 nm and the content of sugar was reduced.

Leite stated B-glucosidase (βGLase), 0.5 mL crude and 1.0 mL cellobiosis dissolved at 0.05 M citratephosphate buffer pH 4.8, 50 tonnes of chicken for 30 mins. The DNS technique was used to monitor glucose emissions.

Statistical Analysis

The mean ± standard deviation (SD) (n = 3) of all data were expressed. ANOVA calculated the obvious differences. The $p < 0.05$ were considered to be significant.

4. RESULTS AND DISCUSSIONS

Chemical characterization of the substrates

Table 2 shows the chemical and basic content of untreated and processed bagasses (using acid-alkaline and hydrogen peroxide pretreatments).

Table 2

Chemical and elementar compositions of the untreated and pretreated bagasses.

	Natural sugarcane bagasse	Pretreated bagasse acid-alkaline	Pretreated bagasse hydrogen peroxide
Moisture (%)	5.37 ± 0.25	6.85 ± 2.07	0.28
Ash (%)	0.73 ± 0.02	2.13 ± 0.10	1.73 ± 0.10
Extractives (%)	13.96 ± 2.53	13.13 ± 1.12	10.64 ± 0.49
Lignin (%)	15.61 ± 3.72	8.23 ± 1.24	10.56 ± 0.29
Hemicellulose (%)	28.75 ± 2.99	2.71 ± 0.30	22.37 ± 0.97
Cellulose (%)	35.92 ± 0.25	75.01 ± 0.36	47.23 ± 0.78
%C	45.79	43.08	44.50
%H	6.23	5.97	6.01
%N	0.53	0.29	0.40
%S	1.08	0.99	1.01
%O	44.24	48.94	45.69

The findings indicated that up to 47.3 percent and 90.6 percent of lignin and hemicellulose were eliminated in acid-alkaline pretreatment. However, only 32 and 22.3 percent of lignin and hemicellulose were eliminated when hydrogen peroxide was employed in pretreatment. Similar results were made by Rezende et al. (2011) who utilized sugarcane bagasse pretreatment with acid-alkaline (NaOH 2 percent or higher). In contrast, 55% of lignin from sugar cane bagasse has been taken from Guilherme et al. (2015) utilizing both acid-alkaline and hydrogen peroxide pretreatments. However, hemicellulose removal values were lower in this instance than obtained in this research. These findings indicated that acid-alkaline pretreatment is more effective to delign the sugar cane bagasse and therefore reduces the recalcitrance of the lignocellulosic residue. Lignin is regarded as the primary contributor to biomass recalcitrance, but other variables such as hemicellulose content and high acetyl content in xylene chains also interfer.

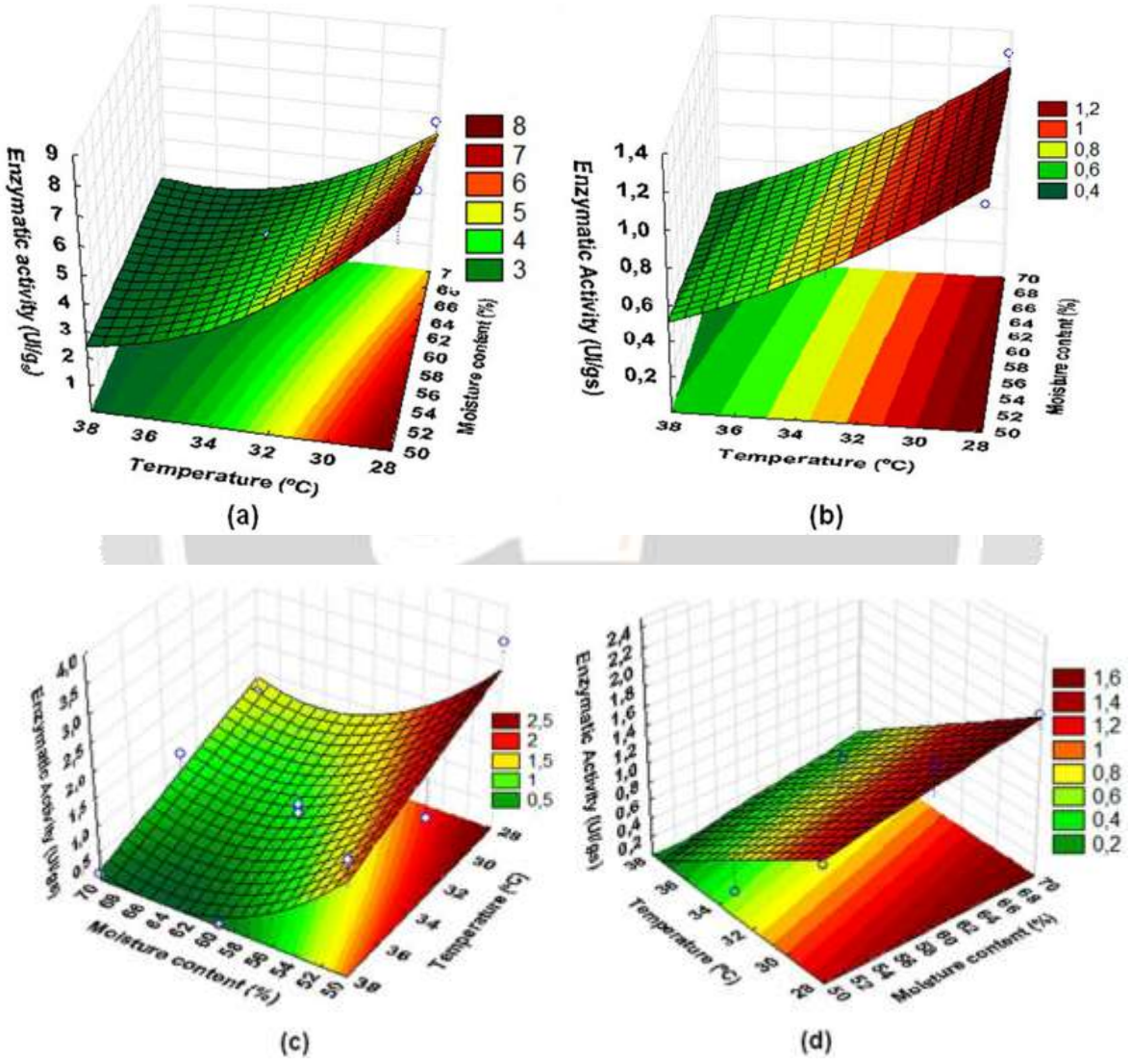
Enzymatic production

Cellulases were manufactured using Rhizomucor sp., Penicillium sp. and Trichoderma koningii fungus. The Rhizomucor sp. fungus. Penicillium and sp. Sp. The grease trap scum situated in Vitória, ES, Brazil was isolated. The objective was to check if these fungal strains in the area are potential cellulase producers. The literature says that Penicillium species may be excellent cellulase producers (Ritter et al., 2013; Behera and Ray, 2016), which has not been seen in Rhizomucor. Trichoderma is one of the most frequent manufacturers of commercially available cellulases, and was utilized for comparison with other microorganisms in this study.

Enzyme (IU/gsubstrate) activity derived from the SSF of T. Penicillium sp. Koningii Rhizomucor and sp. Table 3 is given. The enzyme production factors assessed were temperature (28, 33 and 38 °C) and humidity (50 percent, 60 and 70 percent), the natural sugarcane bagase (NSB) and pre-treated bagasses with acid-alkaline solution (AAB) as well as hydrogen peroxide (HPB). In a review conducted by Paramjeet et al. (2018), culture temperatures from 25 °C to 45 °C may be seen when utilising fungal strains.

Temperature and moisture impact on the synthesis of the enzymes were examined statistically and the findings are given in Table 4, where only statistically important factors are reported ($p < 0.1$). The Rhizomucor sp SSF. The enzyme activity was extremely low and the statistical analysis of these data was thus not conducted. The statistical examination of the information revealed that the residues were randomly and independently distributed and a normal distribution followed, along with excellent correlation coefficients for all suggested models. This allowed the modified models to represent the data. Some planned experiments were excluded for the experimental analysis: run 2 to Trichoderma koningii and NSB, run 4 to T. Run 2 to Penicillium sp, koningii wit AAB and HPB. And NSB, since the residue analysis has identified outlier.

Statistical study revealed that humidity and temperature interfered with Trichoderma Koningii's enzyme synthesis utilising Eqs as substrates NSB, AAB, and HPB. 1, two and three respectively. The temperature impact was greater with NSB and AAB as substrates, but was somewhat lower with HPB. The answer emerges for the tests with T. The koningii substrates NSB, AAB, HPB are illustrated correspondingly in the Figs. 1a, 1b, 1c, 1c. The larger enzymatic activity may be seen at lower temperatures and lower humidity levels (28 °C and 50 percent). Statistical study revealed that the moisture does not interfere with the synthesis of the enzyme for Penicillium sp. only natural bagasse, with an impact lower than that of Temperature. One potential reason is that the structural component of the native bagasse which has partly been removed from the treated bagasse would absorb more water. Figure 1d indicates that lower temperatures (28°C) and greater humidity levels (70%) resulted in improved enzyme synthesis when NSB was employed. The use of AAB enhanced the synthesis of the enzyme at intermediate temperatures (Figure 1e), where the optimal temperature is 32 °C. The optimal temperature in tests using HPB was 29 °C (Figure 1f).



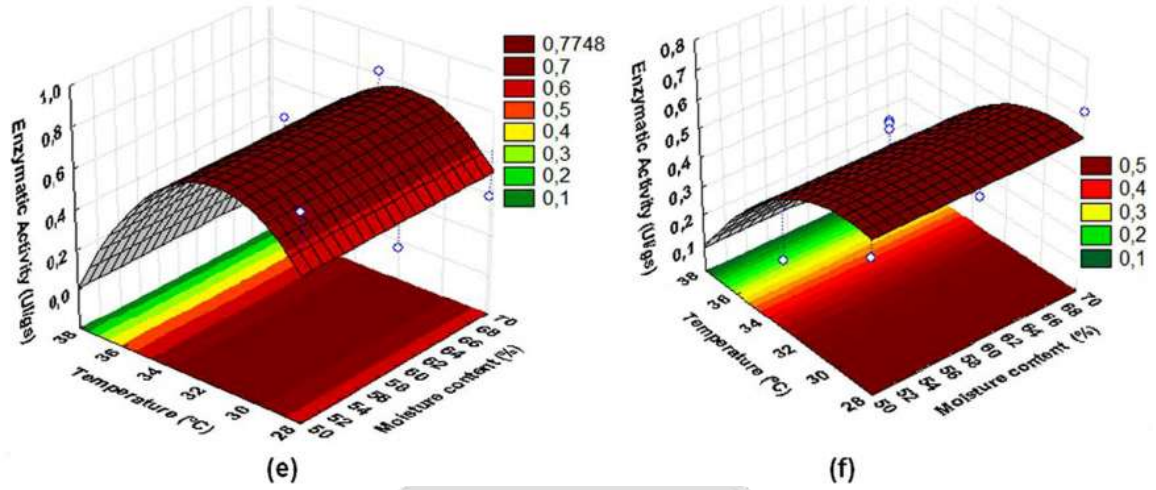


Figure 1: Response surfaces for enzyme production by SSF of Trichoderma koningii using NSB (a), AAB (b) and HPB (c); and SSF of Penicillium sp. using NSB (d), AAB (e) and HPB (f).

5. CONCLUSION

The fungus Rhizomucor exhibited minimal cellulase production. Although the activity levels of Penicillium were better, their production was less than Trichoderma koningii, which is supported by literature data. With regard to crop substrates, it was confirmed that sugar cane bagasse can be utilized to produce the cellulases and as a carbon source for investigated microorganisms. When bagasse was not processed, the best output results were achieved. The temperature had a larger impact on the synthesis of enzymes among the operational factors examined, leading to better outcomes when utilizing temperatures between 28 and 30 °C.

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