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An Enrichment of Xylanolytic Organism with High pH Optima

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Abstract: The treatment of straws from different plants for a long time up to 60 days could lead to an enrichment of organisms which have xylanases with high pH optima. The isolated fungi from ammonia treated-rice staw and sugar cane bagasse recorded 366 colonies constituting 10 species, while sugar can bagasse isolates reached 215 colonies representing 7 species. Sugar can bagasse as an isolation source possessed higher fungal diversity than rice straw. Mesophilic fungi were dominant in either rice staw or sugar can bagasse sources followed by the thermophilic fungi, which were isolated rarely. Scopulariopsis brevicaulis highly dominated straw and bagasse. Thermophilic *Aspergillus versicolor* and mesophilic *Scopulariopsis brumptii* dominated bagasse, whereas thermophilic *Aspergillus terreus* and mesophilic *Acremonium strictum* were encountered from straw.

Key words: Xylanolytic organisms, pH, thermophilic, natural products

INTRODUCTION

The alkaline cooking liquor to a pH value lower than 8.5 and the concomitant corrosion during the enzyme treatment is the biggest single problem. Against this background, the isolation of organisms, which have xylanases with high pH optima, is of high importance for this field of biotechnology.

Xylanase are of great interest to the pulp and paper industry due to their bleach boosting properties helping to replace the use of damaging bleaching agent. A bleach booster in an enzyme facilitating lignin to be removed advantageous working conditions for a bleach booster is high temperature and alkaline conditions. Therefore, an ideal xylanse should be able to function under extreme condition[1].

Puchart *et al.*[2] found a group of 17 stains of the thermophilic fungus *thermomyces lanuginosus* for the production of xylanases. All strains were found to be xylanolytic and several were proven to be outstanding producers of microbial xylanase on glucuronoxylan and corncobs. The stains hyper-producing xylanase secreted low amounts of xylan disbranching enzymes. Oily the strains showing lower xylanase production exhibited a higher degree of xylan utilization. Some of the stains have good potential for use as sources of important industrial of high thermal stability.

Tsiklauri *et al.*[3] showed that *Funalia trogii* 0146 is an active producer of xylanase, which was, selected from 15 strains of higher basidial fungi by submerged and solid phase fermentation. Extracellular enzyme activities of basidiomycetes were demonstrated to vary considerably in fungi of various taxonomic and ecological groups and depend on the plant sub substrate in the medium and the method of macromycete cultivation. Experiments with various carbon sources in the medium showed that xylanase of *F. trogii* 0146 are inducible enzymes.

Thermomyces lanuginousus strains from different culture collection were compared for xylanase production and isozyme profile. All strains were found to produce two forms of xylanase (I, II) with molecular weight corresponding to 25.0 and 54.0 K.Da.[4].

An extracellular xylanase was purified to homogeneity from the culture filtrate of a thermophilic fungus, *thermomyces launginousus*-SSBP. The optimal pH of xylanase activity was 6.5 and the enzyme appeared to be stable over a broad pH range (pH 5-12) under the assay conditions. This xylanase has a potential use un biopulping processes and other industrial applications[5].

When production of xylanases by 4 thermophilic fungi was studied *Chrysosporium sulfureum, Penicillium purpurogenum, Aspergillus terreus* and *A. fumigatus* differed significantly in the degree of production of xylanases. *P. Purpurogenum* was an efficient producer of xylanase[6].

Bhatt *et al.*[7] found that *Aspergillus niger* exhibited maximum xylanase activity in liquid medium containing iarch wood xylan at 28°C and pH 5.

Two different xylanases, CX -I and CX - II from an alkalophilic fungus, *Cephalosporium* sp. Strain PYM 202, have been purified to homogeneity[8].

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Four xylanase preparations from different microorganisms were described. A xylanase from *Thermomyces launginosus* (DSM 5826), *Schizophyllum commune* (BT 2115) and *Penicillium simplicissimum* (BT 2246) were isolated[9].

Gomes *et al.*[10] found that xylanases produced by several strains of *Aspergillus, Gliocladium, Schizopyllum and Trichoderma* have very poor hydrolytic efficiencies. Other fungal cultures *Sporotrichum thermophile*, *Trichoderma reesei*, *Penicillium sp.*, *Thielavia terrestris*, *Sporotrichum cellulophilum* and *Talaromyces emersonii* are solo poor producers of xylanase alone with different components of cellulase[11].

A xylanase producing alkalophilic Bacillus NT-9 was obtained by screening methods of transparent zone on the selective medium[12]. An alkalopphile *Aspergillus nidulans* kk99 produced an alkaline thermostable xylanase[13]. And akaophilic and xylanolytic bacterium, strain SM-XY 60, was isolated from the gut of a higher termite *Sinocapritermes mushae*[14].

The main aim of the present study was to reach for new sources of *Thermophilic alkalophilic* fungi, which have xylanases with high pH optima, is done by prolonging the ammonia- treatment of straw from different plants up to 60 days. This approach is highly innovating.

A survey of the literature in Biological abstracts and current contents revealed that such an approach was not taken so far (or not published in a manuscript with proper key word index).

MATERIALS AND METHODS

Source of isolation: The microorganisms recorded in the study were isolated from rice straw and sugar cane bagasse, collected from after soaking in water for 3 days and then fermenation was carried out by spraying with water and ammonia solution to prepare a humid and alkaline media.

Four isolation experiments were performed every 15 days for two months and the resulting fungal species were identified and kept on slants for further studies.

Fungal isolation from rice staw and sugar cane bagasse: One gram each of sugar cane bagasse and rice straw was added to a tube of 10 mL distilled water and then 1 mL from each tube was taken and added to a tube of nine mL distilled water (dilution 1:10) and then introduced into a shaker for 30 min. Xylan medium was selected and used as isolation medium. It has the following composition (g L^{-1}) xylan, 10 K; pepton, 5; yeast extract, KH_2PO_4; $MgSO_4$. $7H_2O$, 0.5; agar, 20; increased to 30 in case of thermophilic fungi. The medium was brought up to 1000 mL with distilled water and the pH was adjusted at 10 by addition of carbonate bicarbonate buffer solution, which was sterilized separately. After solubilization and sterilization by autoclaving, streptomycin 30 μg mL^{-1} were added. Fifteen milli liter of this medium in case of mesophilic fungi or 30 mL in case of thermophilic fungi were cool to just above the solidification and added to each petridish. One milliliter from each tube of sugar cane bagasse and rice straw were transferred aseptically into each of three petri dishes containing isolating medium, the dishes were rotated by hand in a broad swirling motion so that the diluted samples were dispered in agar.

After incubation at 27°C (for mesophilic fungi) or at 45°C (for thermophilic fungi)[15-17] for 7 days, the resulting colonies were counted and identified. Fungi which grew at both incubation temperature (27 and 45°C) were referred to as thermotolerant.

Identification of fungi: The developing fungal colonies were identified up to the species level by microscopic examination. This was made through the help of the following: Barnett[18], for the genera of imperfect fungi; Barnett and Hunter[19], for imperfect fungi; Barron[20], for the genera of hyphomcetes; Ellis[21-22], for Dematiaceous hyphomycetes; Gilman[23], for soil fungi; Kendrick[24], for imperfect fungi; Moubasher[25], for soil fungi in Qatar and other Arab countries; Raper and Fenell[26], for *Aspergillus genus* and Samson[27], for *Aspergilli* described since 1965.

RESULTS

The microfloral picture of rice straw and sugar cane bagasse: The rice straw and sugar cane bagasse samples were assayed after fermenation with ammonia for 15 days in four isolation per two months. Mycological surveys of samples were carried out thereafter to estimate the total fungal count and density percentage of isolated species. The fungal species were classified into mesophilic fungi (grown at 27°C) and thermophilic fungi (grown at 45°C).

Table 1 includes the total counts and numbers of isolated fungal species from both treated rice straw and sugar cane bagasse sources every fifteen days for two months. A total of 366 fungal colonies were isolated all over the experiment.

The mesophilic fungi counted 353 colonies, which represented 96.4% of the total count and the thermophilic fungi 13 colonies that accounted for 3.6%. Eleven fungal species were totally isolated (*Scopulariopsis brevicaulis* occurred two times on both sources) of which 9 species were mesophilic and 2 were therophilic.

Table 1: Total counts and numbers of isolated fungal species from both treated rice straw and sugarcane bagasse sources every 15 days for 2 months (I, II, III and IV)

		Isolation										
		I		II		III		IV				
Source of isolation		Mes.	Thph.	Mes.	Thph.	Mes.	Thph.	Mes.	Thph.	Total Mes.	Total Thph.	Total
Rice straw	Count	-	-	29	-	40	-	72	10	141	10	151
Fungi	No. of spp.	-	-	2	-	3	-	3	1	3	1	4
Sugar cane	Count	-	-	10	-	30	-	172	3	212	3	215
Bagasse Fungi	No. of spp.	-	-	1	-	1	-	6	1	6	1	7
Total	Count	-	-	39	-	70	-	244	13	353	13	366
	No. of spp.	-	-	3	-	3	-	9	2	9	2	11

Table 2: Surveys of genera and species as well as the Relative Densities (RD%) of fungi isolated from rice straw and sugar cane bagasse every 15 days isolations for two months (I, II, III and IV)

		I		II		III		IV		Straw		Bagasse		Total	
Temp. response	Fungal species	S	B	S	B	S	B	S	B	Count	RD (%)	Count	RD (%)	Count	RD (%)
Mesophilic fungi	*Aspergillus* spp. (total)	-	-	-	-	-	-	26	26	26	17.22	26.0	12.09	52	
	A. flavus	-	-	-	-	-	-	-	10	-	-	10.0	4.70	10	14.21
	A. niger	-	-	-	-	-	-	-	3	-	-	3.0	1.40	3	2.73
	A. sydowi	-	-	-	-	-	-	26	-	26	17.22	-	-	26	0.82
	A. tamarii	-	-	-	-	-	-	-	13	-	-	13.0	6.05	13	7.10
	Acremonium strictum	-	-	26	-	30	-	43	-	99	65.56	-	-	99	3.55
	Curvularia geniculata	-	-	-	-	-	-	-	13	-	-	13.0	6.05	13	27.05
	Scopulariopsis spp. (total)	-	-	3	10	10	30	3	133	16	10.60	17.3	80.46	189	51.64
	S. brevicaulis	-	-	3	10	10	30	3	23	16	10.60	6.3	29.30	79	21.58
	S. brumptii	-	-	-	-	-	-	-	110	-	-	110.0	51.16	110	30.05
Thermophilic fungi	*Aspergillus* spp. (total)	-	-	-	-	-	-	10	3	10	6.62	3.0	1.40	13	3.55
	A. terreus	-	-	-	-	-	-	10	-	10	6.62	-	-	10	2.73
	A. versicolor	-	-	-	-	-	-	-	3	-	-	3.0	1.40	3	0.82
	Total	-	-	29	10	40	30	82	175	151	100.00	215.0	100.00	366	100.00
Number of species		-	-	2	1	2	1	4	7	4		7.0		10	

S=Rice straw, B= Sugar cane bagasse

The total count of rice straw fungi all over the four isolation was 151 colonies, which constituted 4 species (3 mesophilic and 1 thermophilic). In sugar cane bagasse, higher fungal count (215 colonies) was recorded and composed of 7 species (6 mesophilic and 1 thermophilic).

The highest mesophilic fungal count was recorded in all isolated except only during the fourth isolation period in which 10 colonies were recorded from rice straw and 3 colonies from sugar cane bagasse constituting one species for each sources.

Species range of mesophilic and fungi in rice staw and sugar cane bagasse samples: Table 2 includes surveys of genera and species as well as the Relative Densities (RD%) of mesophilic (grow at 27°C) and thermophilic fungal species (grow at 45°C) isolated from rice straw and sugar cane bagasse samples every 15 days for two months. The Relative Density (RD%) of each genus and species was calculated as percentage as percentage of total count.

Mesophilic fungal species: *Scopulariosis brumtii* was the most frequent mesophilic species. It was isolated once (during the fourth isolation) from sugar cane bagasse and was missed in rice straw samples. It was represented by 110 colonies, which constituted 51.16% of total sugar cane bagasse isolations and 30.05% of the total isolation.

Acreemonium strictum was the second mesopholic fungus in density of the total rice straw isolates (65.56%) and the isolated (27.05%). It was isolated during II, III and IV isolations with a total count of 99 colonies being recovered from rice straw samples only.

Scopuariopsis brevicaulis came next in rank of density of the mesophilic fungi. Its count was 16 colonies out of 151, which represented 10.60% of the total rice straw isolation, while its count was 63 colonies out of 215 accounting for 29.30% of the total sugar cane bagasse isolations. *S. brevicaulis* represented 21.22% of the straw isolates and 7.10% of the total isolates.

Aspergillus tamarii and *Curvularia geniculate* were the next species in rank of density being caught only from

Table 3: Relative Density Range (R.D%) of fungal species isolated from rice straw and sugar cane bagasse samples

Relative density range (%)	Rice straw Fungal species	Suger came bagasse Fungal species
50-100	*Acremonium strictum*	*Scopulariopsis brumptii*
40-50	-	-
30-40	-	-
20-30	-	*S. brevicaulis*
10-20	*Aspegillus sydowi*	-
	S. brevicaulis	-
1-10	*A. terrus*	*A. flavus*
	-	*A. niger*
	-	*A. tamarii*
	-	*Curvularia geniculata*
	-	*A. versicolor*

sugar cane bagasse. Each was represented by 13 colonies out of 215, which accounted for 4.70% of the total sugar cane bagasse isolated and 2.73 of the total isolates. It was isolated once from the fourth sugar cane bagasse sample.

Aspergillus niger was the last and least species in order of density with a count of 3 colonies caught once from the fourth isolation of sugar cane bagasse sample.

Thermophilic fungal species: only two thermophilic fungal species were recorded during the present study with low densities. *Aspergillus terreus* (10 colonies) caught once from the fourth rice straw isolation accounting for 6.62% of the total rice straw isolates and 2.73% of the total isolates and *Aspergillus versicolor* which was also caught once from the sugar cane bagasse sample with a isolates and 0.82 of the total isolates.

Table 3 represents the Relative Density range (RD%) of the isolated fungi from rice straw and sugar cane bagasse samples. The data show that only one species, *Acremonium strictrum*, occupied the high-density range of 50-100% in rice straw samples, whereas *Scopulariosis brumptii* occupied the same range in sugar cane bagasse samples, a density gap not occupied by any fungal species was recorded between 20-30%. The mesophilic fungi *Aspergillus sydowi* and *S. brevicaulis* in rice straw samples occupied the density range of 10-20%.

The Density Range of *A. terreus* and *A. versicolor* representing thermophilic fungi was 110% in rice straw and sugar cane bagasse, respectively. Also four mesophilic fungal species was recorded in the same range on sugar cane bagasse sample namely *A. flavus, A. niger, A. tamarii* and *Curvularia geniculata.*

DISCUSSION

In the present study two plant sources, treated with ammonia for two months, have been chosen to isolated xylanolytic fungi: a) rice straw b) sugar cane bagasse.

Four surveys, each every 15 days for two months, resulted in isolating 366 colonies constituting 10 different species from the two sources. The total isolates from rice straw and sugar cane bagasse were 151 colonies representing 4 species and 215 colonies representing 7 species, respectively. Mesophilic fungi representing 96.4% of the total fungal isolates from the two sources. While thermophilic species accounted only 3 species and it counted 212 colonies on sugar 10 colonies constituting only one species.

Recently, screening of xylan- decomposing filamentous fungi were carried our in Lower Egypt. Twenty-six species representing 13 genera were identified Twenty-three species and eleven genera drom rice straw, sixteen species and seven genera from wheat straw and twenty species and eight genera from sugar cane bagasse were isolated[28].

Also, fungi, comprising about 4000 cultures, were collected from different climatic zones of the southern Caucasus. Almost all cultures in the collection showed a high potential for degrading basic degrading basic plant biopolymers such as cellulose and hemicelluloses. Few of them had high xylanase activities. More than 6% of all cultures in the collection were thermopiles and from these, 56 cultures with the highest xylanase activity were selected. It was shown that under two different thermophilic growth conditions, 40 and 48°C, *Allescheria terrestris* formed endoxylanases with different thermal stabilities. It was also shown that when cultivated on straw, *Allescheria terrestris* grows primarily in its internal part for an extended period of time [29].

A plant agar technique for fungal screening was applied to evaluate the xylanolytic activities of 18 *Penicillium janthinellum* and 10 *Aspergillus sydowi* species from the Amazon region. In order to compare these genera with those of other regions, one *Aspergillus sp.*, one *P. janthinellum* and 12 unknown genera from the southern region of Chile were studied. From these fungi, *A. sydowi* (56 strain) (25.2 unit mL^{-1}) and *P. janthinellum* (671 strain) (47.3 unit mL^{-1}) from Amazon, *P. janthinellum* (X4Z2 strain) (9.5 unit mL^{-1}) and an *Aspergillus sp.* (X2M1 strain) (33.3 unit mL^{-1}) from the southern region of Chile were identified[30].

In the present study, *Scopulariopsis brevicaulis* was the most dominant mesophilic species in the two treated of isolation. The mesophilic *S. brumptti* and thermophilic *Aspergillus versicolor* were isolated at high densities from sugar cane bagasse only; they are specific for sugar cane bagasse. On the other hand *mesophilic Acremonium strictum, A. sydowi* and thermophilic *A. terreus* were the most prevalent on rice straw only. The remaining fungal species *A. flavus, A. niger, A. tamarii* and *Curvularia geniculate* were isolated with relative densities ranging from 1-10% from only sugar cane bagasse.

In this connections, *Aspergillus flavus, A. niger, Penicillium cheysogenum, P. corylophilum, P. funiculosum, P. oxalicum* and *Trichoderma harzianum* were the most prevalent species were dominate on one substrate and less frequent or not recovered from the others[28]. More filamentous fungi were recovered from lack silage on media containing carboxymethylcellulose, pectin, or xylan. The most commonly isolated taxa were *Absidia* sp., *Aspergillus flavus*, *Aspergillus fumigatus*, *Byssochlamys nivea*, *Monascus rubber*, *Penicikkium brevcompactum*, *Pseudoallescheria boydii* and *M. brevicaulis*[31].

Kadowaki *et al.*[32] isolated *Aspergillus tamarii* from soil during a screening program for xylanase producing microorganisms. Also Simão *et al.*[32] succeeded in induction of xylanases from *Aspergillus tamarii* by methylbeta -D-xyloside. Four anaerobic fungi were grown on filter paper cellulose and monitored over a 7-8 days period for substrate utilization, fermentation products and secretion of cellulolytic enzymes. Two of the fungi were *Neocallimastix* species isolated from a ruminant (sheep) and the other two fungi were *Piromyces* species isolated from an Indian elephant and an Indian rhinoceros, respectively. The tested anaerobic fungi degraded the filter paper cellulose almost completely. All strains secreted xylanase enzymes[34].

Surveys of the potential for production of extracellular hydrolytic enzymes by *mycorrhizal* fungi on potato-dextrose liquid medium were determined. Xylanase activities seemed to be higher than those of the other carbohdrases[35].

In the present work, isolated from rice straw sources represented one thermophilic species *(Aspergillus terreus*) and three mesophilic species (*A. sydowi, Acremonium strictum* and *Scopulariopsis brevicaulis*). When sugar cane bagasse was the source, *A. tamarii, Curvularia geniculata geniculata, S. brevicaulis* and *S. brumptii* were the mesophilic species recorded. It appears, therefore, that screening for xylanolytic fungi revealed that sugar can bagasse is a more suitable substrate as the source of xylan than rice straw sources.

Melanocarpus alomyces IIS-68, a thermophilic fungus was used for the production of extracellular xylanase on various agroresidues in Solid-state Fermenation (SSF).

Growth on untreated wheat straw and sugar cane bagasse supported xylanase production; xylanase was produced concurrently with maximal activity being produced on bagasse[35].

Alkali-treated cellulosic and lignocellulosic materials as substrates for cellulase production by *Sporotrichum thermophilia* revealed that the most easily degraded substrate was sugar cane bagasse at 2% concentration. This substrate when alkali treated was the most susceptible to enzymic hydrolysis by culture filtrates of *S. thermophila* grown on untreated bagasse. Optimum Hydrolysis was obtained after 18 hours incubation with the filtrate at pH 3.5-4 and 45°C. Alkali treatment of bagasse reduced its lignin contents significantly and the culture filtrate of *S. thermophila* grown on untreated bagasse contained xylanase[37].

Ortega *et al.*[37] found that when four mushroom strains of *Pleurotus* spp. were cultivated on sugar cane crop residues for 30 days at 26°C, biochemical changes affected the substrate because of fungal growth in terms of nitrogen, lignin, cellulose and hemicelluloses contents. All strains showed variable xylanolytic action.

From the present results, the fungal population was not detected during the first isolated. Mesophilic fungi began to flourish during the fourth isolated period. Thermophilic fungi appeared once during the fourth isolation period. This indicated a positive relationship between fungal proliferation and time of alkali treatment, which might lead to increase in the rate of substate decomposition. Surprisingly, all *Asperigilli* were not recorded expect during the last fermentation period.

Native cellulose is generally resistant to hydrolysis by enzymes or chemical reagents due to the crystallinity and presence of lignin. Other factors such as accessible surface area, pore size distribution, degree of polymerization, dimensions of unit cell and moisture content, also affect the enzymatic and chemicals conversion of cellulose to sugars[39]. The application of various pretreatments to cellulosics can effectively increase the reactivity of cellulose. Various chemical and physical pretreatment methods have been investigated to enhance the rate and the extent of the hydrolysis of cellulose. Pretreatment could be achieved either mechanically or physico-chemically. A mechanical treatment includes compression and grinding in a ball mill[40]. Alkalies and acids are widely used for treatment of the raw materials under different physical conditions. The more frequent agents in this regard are sodium hydroxide, ammonia, sodium hypochlorite, sulfuric acid, phosphoric acid, peracetic acid and hydrochloric acid. Alkali treatment of cellularity is probably the oldest and best-know method of enhancement of microbial degradation of cellulose. In general, the application of alkali results in removal of lignin, an increase in surface area by swelling and alteration of crystalline and amorphous structure[41].

Various types of pretreatment of agricultural and industrial wastes were adopted using many fungi. These include, *Aspergillus terreus*[42], *Aspergillus fumigatus*[43], *Trichoderma viridi*[44] and *T. reesei*[45]. Alkali treatment

and acid chlorite treatment of rice straw and rice husk substrates lead to extensive delignification and enhanced xylanase production[36].

Tanaka[46] studied the microbial colonization and its relation to the decomposition of Phragmites communis reed leaf litter in the area of a saline lake from autumn to summer using litterbag method. There was considerable fungal population on the leaves at the beginning of submergence. These fungi were probably terrestrial in orgin. The fungal population rapidly disappeared in few days after sublergence, when bacteria, including cellulytec and xylanoytic types proliferated. Associated with this rapid colonization of bacteria, decomposition rates of cellulose and xylan increased. The rates decline from day 39 to day 100 with decreasing water temperature, though cellulytic and xylanolytic and xylanolytic fungi increased steeply after day 150. It coincided with a second increase in decomposition rate. Results suggest that the principal decomposer of reed leaf litter were bacteria in the initial phase and fungi in the later phase of the experiment.

In the present work, all isolated fungal species were selected to investigate their extracellular and intracellular xylanase enzyme activities. They *are Aspergillus flavus, A. niger, A. sydowi, A. tamarii, Acremonium strictum, Curvalaria geniculate, Scopulariopsis brevicaulis* and *S. brumptii.*

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