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Production of Cellulase and Xylanase by *Aspergillus niger* KKS

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A fungal strain capable of producing extracellular cellulase was isolated from farmland. It was identified as *Aspergillus niger*, and named *Aspergillus niger* KKS. Production of cellulase and xylanase by the *A. niger* KKS was studied through a shake-flask culture. The effects of culture conditions such as inoculum size, temperature, pH, and medium composition on the cellulase and xylanase production were examined. The optimum temperature and pH for the enzyme production were 30°C and pH 7.0, respectively. The optimized medium was composed of 2.0% (w/v) rice straw, 0.5% (w/v) proteose peptone, 0.5% (w/v) KH_2PO_4, 0.05% (w/v) yeast extract, 0.01% (w/v) $CoSO_4 \cdot 7H_2O$, and 0.05% (w/v) $CuSO_4 \cdot 5H_2O$. When the strain was incubated with the optimized medium, it gave the activities of endoglucanase, β-glucosidase, β-xylosidase, xylanase were 3.80, 4.20, 4.00, 80.0 (IU/mL), respectively. Filter paper and cotton activities were 0.68 and 0.045 (IU/mL), respectively. The results of this study show that *A. niger* KKS is a potential organism with a wide spectrum of enzyme activities, such as those of β-glucosidase, β-xylosidase, and xylanase.

With the increasing shortage of petroleum resources, interest in the bioprocesses for converting renewable cellulosic biomass into useful materials such as liquid fuels and foods have started to increase. For a long-term solution to the energy, chemical and food, resource problems, cellulosic biomass such as agricultural and forest residues, is the only renewable carbon source that is cheaply available in large quantities (1).

Cellulosic biomass has a complex composition. It is composed of cellulose, hemicellulose, and lignin. Most agricultural residues contain 30~50% cellulose, 20~40 % hemicellulose, and 10~20% lignin of dry matter.

Degradation of crystalline cellulose to water soluble glucose and cellobiose is caused by a synergistic action of cellulolytic enzymes (2, 3). Cellulolytic enzymes are generally induced as multienzyme systems. Cellulolytic enzymes have been traditionally divided into three groups, which are endo-1,4-β-D-glucanase (EC 3.2.1.4., 1,4-β-D-glucose glucanohydrolase), exo-1,4-β-D-glucanase (EC 3.2.1.91., exo-cellobiohydrolase, or 1,4-β-D-cellobiohydrolase, CBH), and 1,4-β-glucosidase (EC 3.2.1. 21., cellobiase) (4).

At present, one of the most abundant and inexpensive cellulosic biomass is agricultural waste such as rice straw or wheat straw. Agricultural waste has been considered as the potential substrate because of its high contents of cellulose and hemicellulose and low content of lignin. Industrial and agricultural lignocellulosic wastes are potential sources in the production of ethanol, amino acids, and other useful products by enzymatic methods (5, 6, 7).

Investigations on the cellulose and hemicellulose-hydrolyzing microbial strains to utilize the inexpensive substrate have been done (8). And also a study on the lignin-hydrolyzing strain has been done (9, 10, 11).

The microorganisms which appear to be most promising at present are the *Trichoderma reesei* mutants. However, it was of an interest to us to examine a new microorganism to improve the cellulase and xylanase production.

In this study, our objectives were to isolate the new cellulolytic fungi, to identify the strain, and to examine the optimum conditions for the production of cellulase and xylanase by the isolated strain.

MATERIALS AND METHODS

Microbial Strain

A new strain was isolated during the screening procedure designed to obtain the most efficient cellulolytic fungi.

The basic screening medium was a Malt-Yeast Agar

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(MYA) medium with 50 mg/mL of tetracycline added to inhibit the growth of bacteria. Carboxymethyl-cellulose (CMC) medium-CMC, 10; trypan blue, 0.3; Triton X-100, 1.0; agar, 20 (g/L)-was used to examine the degradation efficiency of cellulose.

To identify the fungal strain, the culture was grown on Czapek's solution agar, Malt extract agar, and Potato dextrose agar at 27°C for 1~2 weeks and the external morphology and feature such as the growth characteristics, mycelium characteristics, colony color, reverse color, etc. of the culture were recorded. The culture grown on malt extract agar for 2~12 days was also observed microscopically.

Culture Maintenance and Inoculum Preparation

The fungal strain was maintained on a malt extract agar slant and transferred monthly. The inoculum for the shake-flask culture was prepared in the following manner : Spore of the culture was inoculated to a malt extract agar slant and incubated at 30°C for 7 days, after which a good spore crop was evident. Five milliliters of sterile distilled water was added to the slant, and the slant was shaken slowly. This spore suspension was used to inoculate starter culture flasks containing 2% (w/v) malt extract. The starter culture flasks were incubated in a rotary shaking incubator at 200 rpm, 30°C for 2 days. Five milliliters of aliquots were taken to be used as the inoculum for further experiments.

Media and Shake-flask Culture Conditions

The basal medium was prepared using distilled water. It contained (g/L): ground rice straw, 20; bacto peptone, 5; KH_2PO_4, 5; yeast extract, 0.5; and $MgSO_4 \cdot 7H_2O$, 0.5. The initial pH of the medium was 7.0. 5% (v/v) of the starter culture aliquot was inoculated in shake-flask cultures. Shake-flask cultures were carried out in 250 mL Erlenmeyer flasks containing 100 mL of the basal medium. The inoculated flasks were shaken continuously on a rotary shaking incubator operating at 200 rpm, 30°C for 7 days unless stated otherwise. The culture broth was filtered through a glass-fibre filter and the filtrate was used as crude enzyme solutions for the assay of enzyme activity.

Enzyme Assay

Endoglucanase, cotton, filter paper, β-glucosidase, β-xylosidase, and xylanase activities were assayed using 1% (w/v) CMC, absorbent cotton (50 mg), Whatman No. 1 filter paper (50 mg), 1 mM p-nitrophenyl-β-D-glucopyranoside, 10 mM p-nitrophenyl-β-D-xylopyranoside, and 2% (w/v) birch wood xylan suspension, respectively, in a 50 mM citrate buffer of pH 4.8, as described by Mandels and Weber (12) and Yu *et al.* (17).

Reducing sugars produced as the result of these assays were determined by using the dinitrosalicylic acid (DNS) method of Miller (13). p-Nitrophenol production was determined from the assays of β-glucosidase and β-xylosidase. Enzyme activity has been expressed in International Units (IU), as the amount of enzyme needed to release 1 μM of glucose, xylose or p-nitrophenol.

RESULTS AND DISCUSSION

Identification of Strain

A cellulolytic microorganism that has a wide spectrum of enzyme activities for utilizing lignocellulosic materials was isolated and identified as *Aspergillus niger*. It was named *Aspergillus niger* KKS. A microphotograph of conidial heads and conidiophore of the culture is shown in Fig. 1.

The culture showed the following characteristics of growth and morphology:

1. In Czapek's agar, 25°C. Colonies are consisting of a compact white or yellow basal felt mycelium, sporulating in abundance with agar, dark brown to black in conidial areas; reverse colorless to pale yellow, wrinkled.

2. In malt extract agar, 25°C. Colonies are spreading broadly and loosely with dense sporulation, black conidial structure; reverse colorless to pale yellow.

3. In potato dextrose agar, 25°C. Colonies are spreading broadly and compactly, growing rapidly, heavy sporulation, conidial areas black; reverse colorless to pale yellow.

4. Conidial heads are large, radiate, later splitting into columns with agar. Conidiophore stipes smooth-walled, variable in length, 13~15 μm in diameter. Phialides borne on metulae, 5~7×3~4 μm. Metulae is 12~20×4~5 μm. Conidia globose to subglobose, 4~5 μm in diameter, with spins and ridges.

Effect of Culture Temperature

The production of enzyme by *A. niger* KKS was exa-

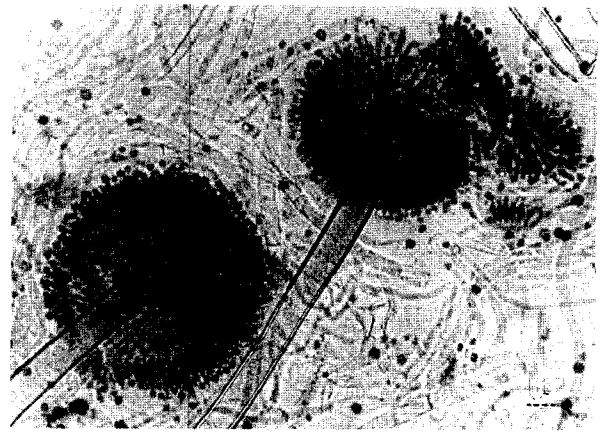

Fig 1. Microphotograph of *Aspergillus niger* KKS showing conidial heads and conidiophore (×500).

Table 1. Effect of temperature on the enzyme production

Temp.	Time (Day)	Final pH	Enzyme Activities (IU/mL)					
			CMC-ase	FPA	Cotton Activity	β-Glu-cosidase	β-Xylo-sidase	Xylanase
30°C	5	3.50	1.2	0.36	0.057	1.1	0.8	8.99
	6	3.44	1.5	0.32	0.055	1.9	1.3	8.65
	7	3.68	2.3	0.49	0.057	3.8	2.2	9.32
	8	3.97	2.1	0.54	0.079	3.8	2.7	9.99
	9	3.79	1.6	0.64	0.063	2.7	1.9	9.32
37°C	5	3.98	2.1	0.45	0.074	2.4	2.0	7.99
	6	3.76	1.5	0.52	0.082	2.3	2.2	8.65
	7	3.83	2.3	0.49	0.084	2.7	2.8	8.66
	8	4.45	1.6	0.46	0.049	1.8	1.8	9.65
	9	5.30	1.6	0.41	0.048	2.8	2.8	10.30

Culture was carried out at 30°C and 37°C for 7days in the basal medium.

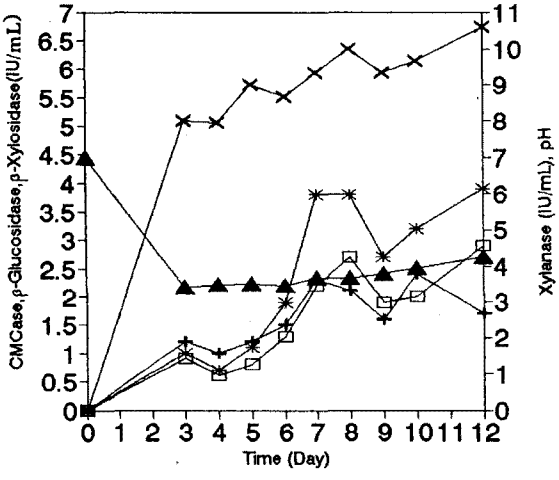

Fig. 2. Enzyme production by *A. niger* KKS from ground rice straw in the shake-flask culture at 30°C.
Straw concentration, 2.0% (w/v). + CMCase, ✳ β-Glucosidase, ⊟ β-Xylosidase, ✕ Xylanase, ▲ pH.

mined at 30°C and 37°C for different periods of time (Table 1).

Maximum enzyme activities were reached in 7~9 days, at 30°C and 37°C. Endoglucanase and FP activities at 30°C were similar to those at 37°C. Cotton and β-xylosidase activities increased 47% and 27% at 37°C compared to those at 30°C, but β-glucosidase and xylanase activities increased 41% and 8% at 30°C compared to those at 37°C. Considering the importance of β-glucosidase and xylanase to this organism, the optimum temperature was determined as 30°C.

Enzyme production by *A. niger* KKS from ground rice straw in the shake-flask culture at 30°C is shown in Fig. 2 and Fig. 3. The pH was decreased from the initial pH of 7.0 to the final pH of 3.4~4.0. The maximum activities of all enzymes were obtained in about 7~8

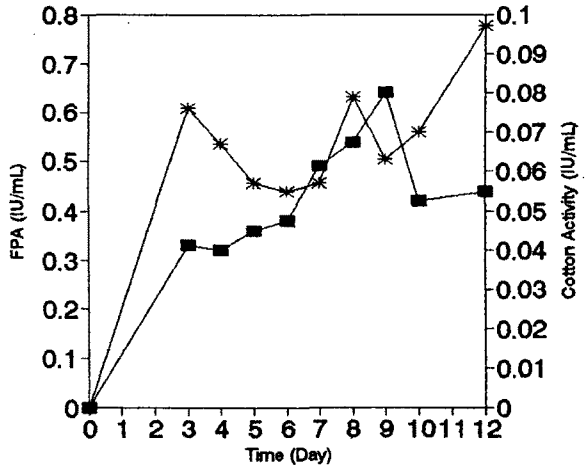

Fig. 3. Enzyme production by *A. niger* KKS from ground rice straw in the shake-flask culture at 30°C.
Straw concentration, 2.0% (w/v). ■ FPA, ✳ Cotton Activity.

Table 2. Effect of initial pH on the enzyme production

Initial pH	Final pH	Enzyme Activities (IU/mL)					
		CMC-ase	FPA	Cotton Activity	β-Glu-cosidase	β-Xylo-sidase	Xylanase
3.0	4.25	1.4	0.24	0.027	3.3	1.9	9.3
3.5	3.69	1.1	0.32	0.024	2.6	1.4	10.3
4.0	3.60	1.7	0.15	0.017	1.8	1.0	10.0
4.5	3.34	1.0	0.17	0.016	2.2	1.4	9.0
5.0	3.30	1.6	0.19	0.018	1.9	1.2	10.3
5.5	3.30	1.5	0.22	0.019	1.9	1.2	11.0
6.0	3.64	1.3	0.19	0.018	2.4	1.4	11.3
6.5	3.55	2.2	0.20	0.024	2.1	1.2	11.7
7.0	3.53	2.4	0.38	0.029	2.8	1.8	12.2
7.5	3.80	1.4	0.37	0.028	2.3	1.3	12.1

Culture was carried out at 30°C for 7 days in the basal medium.

days.

Effect of Initial pH of Medium

The initial pH of the basal medium was adjusted to pH 3.0~7.5 with 0.1 N HCl or 0.1 N NaOH. The enzyme activity and final pH of the culture filtrate were measured after 7 days of incubation (Table 2).

In most cases, the final pH values were between 3.3 and 3.8. Gokhale *et al.* (8) have also reported a similar phenomenon in an experiment with *A. niger*.

The remarkable feature on the effect of initial pH is that the enzyme production reaches nearly equivalent level for the all range of initial pH tested. It indicates that *A. niger* KKS is very stable over the wide range of initial pH.

This stability of the enzymes over the range of initial pH are significant in the saccharification process. Maximum yield of most enzymes were obtained when the initial pH of the basal medium was adjusted to 7.0.

Table 3. Effect of inoculum size on the enzyme production

Inoculum size (%, v/v)	Enzyme Activities (IU/mL)					
	CMCase	FPA	Cotton Activity	β-Glu-cosidase	β-Xylo-sidase	Xylanase
loop	1.9	0.46	0.038	2.7	2.0	10.0
5%	1.9	0.56	0.036	3.0	1.8	11.3
10%	1.8	0.46	0.044	2.6	1.6	13.3
15%	1.0	0.46	0.023	1.3	0.7	12.0
20%	1.8	0.51	0.036	1.8	1.0	10.7

Culture was carried out at 30°C for 7 days in the basal medium.

Effect of Inoculum Size

Table 3 shows the effect of inoculum size on the enzyme production.

Although the production of some enzyme components was greater when a loop and 10% (v/v) of inoculum were used, 5% (v/v) of inoculum size was determined to be the most suitable for the enzyme production, considering the importance of FP and β-glucosidase activities.

Effect of Carbon Source

Cellulase production was found to be dependent upon the nature of the carbon source used in the medium. Therefore, the effect of various carbon sources on the enzyme production was investigated and the results are shown in Table 4.

Soluble sugars did not fully suppress the production of cellulase, as they did in the experiment with *A. fumigatus* by Triviedi and Rao (16), and lactose and maltose did not act as inducers as they did in *T. viride* (14).

Ground rice straw was the most effective inducer among the cellulosic materials used, although rice bran was somewhat comparable. Significant amounts of β-xylosidase and xylanase activities were obtained by using xylan, which was similar to those obtained by using rice straw.

When cotton and filter paper was used as substrate in the medium, there were no detectable activities for the all the enzyme tested (data not shown). However, Taniguchi *et al.* (15) reported an increase in the enzyme production when the filter paper was used by *P. filamentosa* as a carbon source.

A pulp produced about 30~50% of enzyme amounts produced by rice straw and Sigmacell type 20 produced only the 10~20% of those produced by rice straw. On the other hand, solka floc BW200 produced considerable amounts of endoglucanase, β-xylosidase, and xylanase even though the activities of those were lower than those produced by rice straw.

Considering the high levels of enzyme activities (particularly xylanase and β-glucosidase) produced from rice bran and rice straw, the native and impure form of cellulose seems to be superior to much purer form of cellulose (Sigmacell type 20 and UK pulp) in the production of enzymes.

Table 4. Effect of carbon source on the enzyme production

Carbon source	Enzyme Activities (IU/mL)					
	CMCase	FPA	Cotton Activity	β-Glu-cosidase	β-Xylo-sidase	Xylanase
Xylose	0.4	0.18	0.008	0.07	0.24	17.7
Lactose	0.1	0.04	0.002	0.05	0.05	1.7
Maltose	0.0	0.03	0.003	0.13	0.05	2.1
Glucose	0.0	0.04	0.001	0.05	0.04	2.0
Arabinose	0.5	0.21	0.010	0.23	0.16	5.0
Cellobiose	0.1	0.04	0.002	0.01	0.04	1.3
Fructose	0.6	0.26	0.010	0.36	0.22	5.9
Sucrose	0.5	0.25	0.009	0.11	0.08	5.5
Glycerol	0.2	0.03	0.001	0.00	0.04	0.8
Starch	0.0	0.02	0.001	0.01	0.05	1.4
β-cellulose	0.4	0.05	0.003	0.23	0.25	5.3
Sigmacell Type 20	0.6	0.05	0.004	0.10	0.09	3.3
Solka floc BW 200	0.9	0.08	0.009	0.63	0.95	16.1
Pulp	0.7	0.07	0.006	0.30	0.46	8.9
Uk pulp	0.6	0.08	0.001	0.50	0.43	8.3
Sawdust	0.3	0.04	0.003	1.20	0.39	5.5
Xylan (birch wood)	0.7	0.08	0.007	0.50	1.18	21.0
Rice hull	0.7	0.12	0.007	0.70	0.74	16.8
Rice bran	0.9	0.15	0.017	1.60	1.20	20.0
Rice straw	1.3	0.23	0.015	1.50	1.22	20.3

Culture was carried out at 30°C for 7 days in the basal medium containing 1.0% (w/v) carbon source.

Table 5. Effect of ground rice straw concentration on the enzyme production

Concentration (%, w/v)	Enzyme Activities (IU/mL)					
	CMCase	FPA	Cotton Activity	β-Glu-cosidase	β-Xylo-sidase	Xylanase
1%	1.11	0.33	0.025	2.23	1.18	35.0
2%	1.55	0.40	0.023	2.92	1.66	44.1
3%	1.11	0.33	0.013	1.92	1.12	35.5
4%	0.93	0.27	0.010	2.70	1.49	52.5

Culture was carried out at 30°C for 7 days in the basal medium.

As a result, among the carbon sources examined, rice straw was the most effective substrate for the production of enzymes. When the test was done again with various concentrations of rice straw (from 1.0% to 4.0% (w/v)), high levels of enzyme activities were obtained at the concentration of 2.0% (w/v) rice straw (Table 5).

Effect of Nitrogen Source

When various organic nitrogen sources were added to the culture medium, the enzyme production fairly

Table 6. Effect of organic nitrogen source on the enzyme production

Organic nitrogen sources	Enzyme Activities (IU/mL)					
	CMCase	FPA	Cotton Activity	β-Glu-cosidase	β-Xylo-sidase	Xylanase
None	0.34	0.25	0.011	0.13	0.13	6.8
Bacto-peptone	0.97	0.35	0.031	1.65	1.31	23.1
Proteose peptone	1.25	0.51	0.036	2.85	2.28	27.6
Yeast extract	1.48	0.37	0.024	1.49	0.88	20.6
Polypeptone	1.16	0.36	0.028	1.72	0.95	26.1
Urea	1.25	0.33	0.026	1.88	1.19	27.4
Glutamic acid	1.26	0.47	0.029	1.77	1.08	32.2
Tryptone	1.02	0.42	0.031	1.77	1.09	26.3

Culture was carried out at 30°C for 7 days in the basal medium containing 0.2% (w/v) organic nitrogen source.

Table 7. Effect of proteose peptone concentration on the enzyme production

Concentration (%, w/v)	Enzyme Activities (IU/mL)					
	CMCase	FPA	Cotton Activity	β-Glu-cosidase	β-Xylo-sidase	Xylanase
None	0.34	0.25	0.011	0.13	0.13	6.8
0.1%	1.13	0.35	0.020	1.19	0.83	15.7
0.2%	1.05	0.46	0.030	1.79	1.26	24.5
0.3%	1.27	0.42	0.031	2.55	1.65	26.2
0.4%	1.33	0.50	0.030	3.12	1.78	34.4
0.5%	1.38	0.47	0.029	3.15	1.69	30.6

Culture was carried out at 30°C for 7 days in the basal medium.

increased. The results are shown in Table 6.

Proteose peptone was superior to Bacto-peptone in the enzyme production. Since, among the organic nitrogen sources added, proteose peptone gave the biggest amount of β-glucosidase, β-xylosidase, and xylanase, it is clear that proteose peptone has a profound effect on the enzyme production (15). Of the various organic nitrogen sources used, the yeast extract gave the maximum production of endoglucanase.

These results suggest that proteose peptone and yeast extract are the most suitable organic nitrogen sources. When the test was done again with various concentrations (0.1%~0.5% w/v) of proteose peptone, 0.5% (w/v) of proteose peptone was found to be the most suitable concentration (Table 7).

Different concentrations of yeast extract were added to the medium containing 0.5% (w/v) proteose peptone. When 0.05% (w/v) of yeast extract was added, the production of endoglucanase and xylanase was higher than that given by the medium without yeast extract, but the production of β-glucosidase, and β-xylosidase were nearly similar to those given by the medium without yeast extract (Table 8).

Table 8. Effect of yeast extract concentration on the enzyme production

Concentration (%, w/v)	Enzyme Activities (IU/mL)					
	CMCase	FPA	Cotton Activity	β-Glu-cosidase	β-Xylo-sidase	Xylanase
None	1.38	0.47	0.029	3.15	1.69	30.6
0.05%	2.22	0.57	0.026	2.92	1.64	41.3
0.1%	1.04	0.40	0.023	2.70	1.38	24.5

Culture was carried out at 30°C for 7 days in the basal medium.

Table 9. Effect of phosphate salts on the enzyme production

Phosphate salts	Enzyme Activities (IU/mL)					
	CMCase	FPA	Cotton Activity	β-Glu-cosidase	β-Xylo-sidase	Xylanase
None	2.64	0.40	0.015	3.09	1.32	22.8
KH_2PO_4	2.86	0.50	0.023	2.27	1.08	46.3
K_2HPO_4	2.18	0.42	0.022	2.91	1.29	36.3
NaH_2PO_4	1.75	0.43	0.019	3.27	1.56	35.0
Na_2HPO_4	2.49	0.43	0.020	3.78	1.76	28.5

Culture was carried out at 30°C for 7 days in the basal medium containing 0.2% (w/v) phosphate salt.

The effect of different inorganic nitrogen sources such as $(NH_4)_2SO_4$, NH_4NO_3, CH_3COONH_4, NH_4Cl, KNO_3, and $NaNO_3$ on the enzyme production was also studied. All inorganic nitrogen sources used had little effect on the enzyme production (data not shown). As a result, no inorganic nitrogen source was used. In fact, the inorganic nitrogen sources had a tendency to decrease the activities of all enzymes except for those of one or two of enzyme components which showed an increase. It was also found that the nitrogen salt suppresses the production of most enzyme components.

Effect of Phosphate Salts

The effect of various phosphate salts on the production of enzymes is shown in Table 9. 0.2% (w/v) of phosphate salt was used for this test. Among the phosphate salts examined, KH_2PO_4 showed about 8% and 50% of increase in the production of endoglucanase and exoglucanase, and about 15~25% of decrease in the production of β-glucosidase and β-xylosidase and, particularly, the production of xylanase increased up to 100%.

When NaH_2PO_4 was added, the production of β-glucosidase and β-xylosidase increased 6% and 20%, respectively, but the production of other enzymes decreased. When Na_2HPO_4 was added, the production of β-glucosidase and β-xylosidase increased 18% and 30%, respectively, but the production of other enzymes decreased.

Consequently, KH_2PO_4 was found to be the most suitable phosphate salt. When the test was done again

Table 10. Effect of KH_2PO_4 concentration on the enzyme production

Concentration (%, w/v)	Enzyme Activities (IU/mL)					
	CMCase	FPA	Cotton Activity	β-Glu-cosidase	β-Xylo-sidase	Xylanase
None	0.73	0.25	0.007	1.77	0.71	21.4
0.1%	2.07	0.40	0.011	2.37	1.08	48.5
0.2%	1.69	0.38	0.009	1.12	0.59	51.7
0.3%	2.04	0.51	0.011	2.15	0.95	54.6
0.4%	2.02	0.52	0.011	2.61	1.28	57.2
0.5%	1.99	0.50	0.013	2.84	1.59	58.4
0.6%	1.94	0.59	0.013	2.60	1.43	65.5

Culture was carried out at 30°C for 7 days in the basal medium.

Table 11. Effect of metal ions on the enzyme production

Metal ions	Enzyme Activities (IU/mL)					
	CMCase	FPA	Cotton Activity	β-Glu-cosidase	β-Xylo-sidase	Xylanase
KCl	2.58	0.49	0.031	2.28	1.36	56.4
$CuSO_4 \cdot 5H_2O$	1.02	0.47	0.024	1.51	0.99	66.5
$FeSO_4 \cdot 7H_2O$	1.18	0.52	0.027	2.34	1.36	41.0
$ZnSO_4 \cdot 7H_2O$	0.97	0.47	0.028	2.00	1.27	43.4
$CaCl_2 \cdot 2H_2O$	1.65	0.49	0.036	2.12	1.30	43.8
$CoSO_4 \cdot 7H_2O$	1.85	0.56	0.033	2.30	1.56	51.3
$CoCl_2 \cdot 6H_2O$	2.70	0.63	0.039	2.33	1.49	50.5
$MnSO_4 \cdot 5H_2O$	1.63	0.47	0.033	2.34	1.38	55.9
$MgSO_4 \cdot 7H_2O$	1.48	0.49	0.035	2.31	1.43	51.4

Culture was carried out at 30°C for 7 days in the basal medium containing 0.05% (w/v) metal ion.

with various concentrations of KH_2PO_4, 0.5% (w/v) of KH_2PO_4 was found to be the most suitable concentration for the enzyme production (Table 10).

Effect of Metal Ions

The effect of various metal ions is shown in Table 11.

Of the various metal ions used, $CoCl_2$ gave the best production of enzymes. When $CoSO_4$ was added, the production of β-xylosidase and FPA was 10% and 14% more than those given by $MgSO_4$. When $CuSO_4$ was added, the production of xylanase was 29% more than that given by $MgSO_4$.

The effect of various concentrations of $CoCl_2$, $CoSO_4$, and $CuSO_4$ on the enzyme production also studied. Table 12 shows the results of the study. When the overall production of enzymes is considered, the most suitable concentrations of $CoSO_4$, $CoCl_2$, and $CuSO_4$ are 0.01%, 0.03%, and 0.05% (w/v), respectively. In the case of combined metal ions, the combined metal ion of 0.01% (w/v) $CoSO_4$ and 0.05% (w/v) $CuSO_4$ gave the highest production of enzyme (data not shown).

From the above results, the optimized medium was composed of 2.0% (w/v) rice straw, 0.5% (w/v) proteose peptone, 0.5% (w/v) KH_2PO_4, 0.05% (w/v) yeast extract, 0.01% (w/v) $CoSO_4 \cdot 7H_2O$, and 0.05% (w/v) $CuSO_4 \cdot 5H_2O$. Table 13 shows the results when the strain was incubated with the optimized medium.

Table 12. Effect of $CoSO_4$, $CoCl_2$ and $CuSO_4$ on the enzyme production

Concentration (%, w/v)		Enzyme Activities (IU/mL)					
		CMCase	FPA	Cotton Activity	β-Glu-cosidase	β-Xylo-sidase	Xylanase
None		2.10	0.50	0.028	2.51	1.31	53.9
$CoSO_4$	0.01%	3.29	0.53	0.034	2.52	1.49	56.1
	0.03%	2.36	0.58	0.033	1.96	1.29	45.4
	0.05%	2.38	0.53	0.028	1.98	1.46	54.1
	0.07%	2.10	0.47	0.026	0.85	0.79	70.7
$CoCl_2$	0.01%	2.26	0.52	0.034	2.10	1.36	58.9
	0.03%	4.13	0.59	0.042	3.10	2.12	67.6
	0.05%	2.92	0.49	0.031	1.82	1.29	53.8
	0.07%	2.55	0.56	0.032	2.21	1.46	69.4
$CuSO_4$	0.01%	3.40	0.57	0.026	1.47	1.04	107.3
	0.03%	2.38	0.58	0.032	1.67	1.24	80.7
	0.05%	2.38	0.50	0.033	2.60	1.84	90.0
	0.07%	2.50	0.55	0.032	1.76	1.41	77.4

Culture was carried out at 30°C for 7 days in the basal medium containing $CoSO_4$, $CoCl_2$, and $CuSO_4$, respectively.

Table 13. Enzyme production in the optimized medium

	Enzyme Activities (IU/mL)
CMCase	3.80
FPA	0.68
Cotton	0.045
β-Glucosidase	4.20
β-Xylosidase	4.00
Xylanase	80.0

Culture was carried out at 30°C for 7 days.

Overall, *A. niger* KKS produced a wide spectrum of enzymes when grown on rice straw. This substrate has proved particularly suitable for growth and for enzyme production. Although some reports are presented on the production of cellulase and xylanase of *T. reseei* on wheat straw, the literatures involving enzyme production of *A. niger* based on untreated rice straw as an inexpensive substrate were not found. In conclusion, *A. niger* KKS of present study is an useful organism for the production of cellulase and xylanase by using rice straw as a substrate.

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