Production, Purification, Properties and Application of the Cellulases from a Wild type Strain of a Yeast isolate

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ABBREVIATIONS

Aps	Ammonium persulphate
bp	Base pair
BSA	Bovine serum albumin
CMC	Carboxymethylcellulose
dNTP	Deoxyribonucleotide 5'-triphsphate (N= A,T,G,C)
DNS	Dinitrosalisylic acid
FPLC	Fast protein liquid chromatography
HPLC	High performance liquid chromatography
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PI	Isoelectric point
rpm	Round per minute
SDS	Sodium dodecyl sulphate
Taq	*Thermus aquaticus*
TBE	Tris-boric acid-EDTA
TEMED	N,N,N,N-Tetramethyl ethylenediamine
TRIS	2-Amino-2-hydroxymethylpropane-1,3-diol
O.D.	Optical density
UV	Ultraviolet
v	Volume
wt	Weight

1 INTRODUCTION

Cellulases refer to a group of enzymes which act together to hydrolyze cellulose into soluble sugars. They are distributed throughout the biosphere such as plants, animals and microorganisms. However, cellulases from higher plants such as *Lantana camara* and *Cuscuta reflexa* are mostly involved in fruit ripening and senescence (Chatterjee and Sanwal, 1999). Few animals such as the blue mussel *Mytilus edulis* (Bingze et al., 2000), the green mussel (Marshall, 1973), the edible snail *Helix pomatia* (Rebeyrotte et al., 1967; Maeda et al., 1996) the marine mollusc *Littorina brevicula* (Purchon, 1977), termites and protozoa (König et al., 2002) were reported as cellulase producers. Protozoa such as *Epidinium caudatum* and *Eudiplodinium ostracodinium*. Archaea such as *Sulfolobus solfataricus* (Moracci et al., 2001) and *Pyrococcus furiosus* (Voorhorst et al., 1999) are also cellulases producers. However, microorganisms are considered to be the main source for cellulases with novel and high specific activities. Microbial cellulases are the most economic and available sources, because microorganisms can grow on inexpensive media such agriculture and food industries by-products.

It is now well established that the cellulolytic activity is a multicomponent enzyme system and consists of three major components; endo-ß-glucanase (EC 3.2.1.4), exo-ß-glucanase (EC 3.2.1.91) and ß-glucosidase (EC 3.2.1.21).

They have the following specificity:

1. Exoglucanase (1,4-ß-D-glucan) acts on the ends of cellulose chains to produce cellobiose as the main product.
2. Endoglucanase acts randomly on cellulose to produce oligosaccharides of variable sizes (Beguin and Aubert, 1994; Miyamoto, 1997)
3. ß-glucosidases may be divided into three subgroups on the basis of substrate specificity:
   (a) Cellobiases, which hydrolyze only oligosaccharides
   (b) Broad-specificity-ß-glucosidases exhibit activity on many substrate types. They are the most commonly observed ß-glucosidases.
   (c) Aryl-ß-glucosidases, which have a strong affinity for aryl-ß-glucosides (Riou et al., 1998).

Cellulosic materials, its derivatives and polymers with glycosidic linkages are substrates of cellulolytic enzymes. Cellulose is the most abundant organic biopolymer on earth with an estimated annual production of 180 billion tons in nature (Amor et al., 1995; Delemer, 1999). However, its usage depends upon its hydrolysis to available saccharides. Cellulose can be converted into glucose by either chemical, physical treatments or enzymatic hydrolysis. Acid or high temperature degradation is unsatisfactory, because the resulting sugars are partly decomposed.

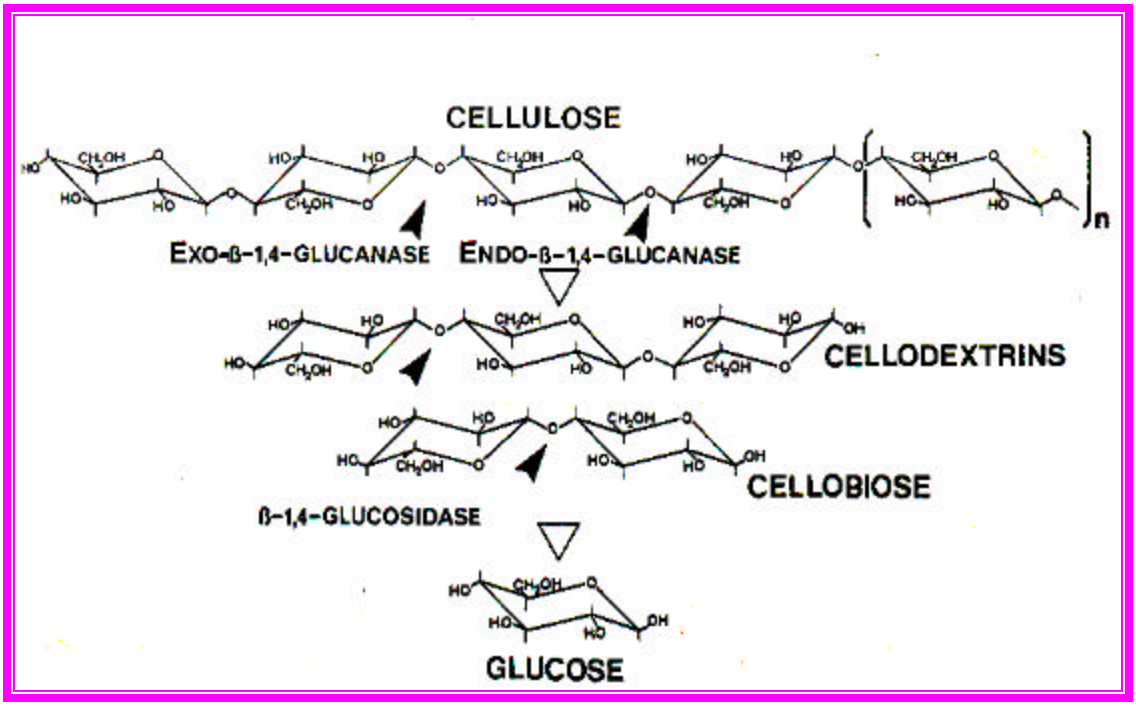

Fig. 1. Cellulose hydrolysis by a cellulase-system.
(Winkelmann, 1992)

Also the cellulosic wastes contain impurities that may generate unwanted by-products such these harsh conditions (He et al., 2000).

Biological degradation of cellulose by cellulases is preferred for industrial purposes due to the high yields of desired hydrolytic products with minimal by-products (Parry et al., 2001). Effective utilization of cellulosic material through bioprocesses will be an important key to overcome the shortage of foods, feed and fuels, which the world may face in the near

future, because of the explosive increase in human population (Ohmiya et al., 1997).

Fig. 2. Examples of agricultureal crops with high cellulosic biomass.
(a. rice, b. sugarcane, c. maize)

The development of economically feasible technologies for cellulase production and for the enzymatic hydrolysis of cellulosic materials will enable us to utilize the large quantities of biomass such as the residues of both food industries and agriculture. Instead, cellulosic material is often burned to clean the fields after harvest each year, and subsequently produce air pollution (Miyamoto, 1997).

Fig. 3. Burning of crops residues in fields after harvest.

The plant cell wall in forages consists of 24-36 % cellulose, 8-38 % hemicelluloses 4.3-8 % lignin (Winkelmann, 1992). The cellulose polymer is an unbranched, insoluble homopolymer composed of up to 14,000 anhydro-D-glucose units linked together by ß-1,4-glucosidic bonds. Characteristic properties of the cellulose polymer are given by the tendency of individual fibers. Ribbon-like chains aggregate together and form rigid fibrils through

intra- and intermolecular hydrogen bonds with high ordered structure, the fibrils associate in a similar way to form fibers (Winkelmann, 1992).

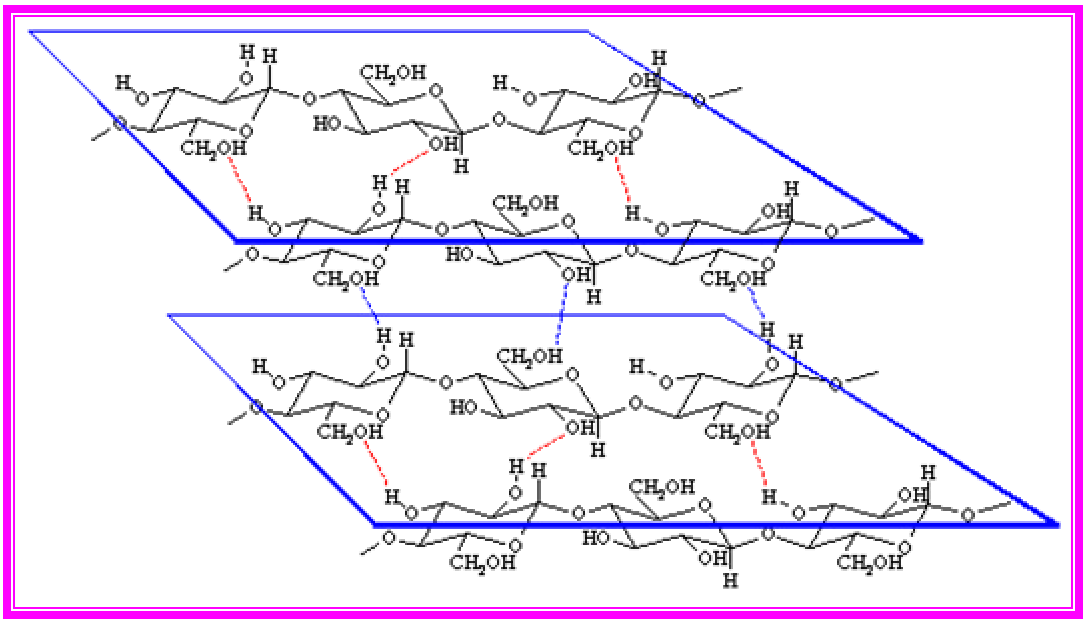

Fig .4. Schematic structures of cellulose chains.

The chains are held together by hydrogen bonds between oxygen of alternating glycosidic bonds in one glucan chain and the primary hydroxyl groups at position 6 of glycosyl residues in another chain. (www.biologie.uni-hamburg.de)

The cellulase enzymes have attracted considerable attention in recent years due to their great biotechnological and industrial potential. Conversions of food industries and agricultures wastes to valuable sugars are the great uses of cellulase enzymes (Bothast and Saha, 1997). The application of cellulase enzyme preparations in food production include the

breakdown of the cellulose in citrus products, increase of the aromatic character of fruit juices through the hydrolysis of flavor glucosidic precursors, decrease the bitterness of citrus juices through the hydrolysis of prunin (Riou et al., 1998), removal of fiber from edible oil press cakes, increase in starch recovery from potatoes, refinement of flour, extraction of proteins from leaves and grasses, tenderizing fruits and vegetables prior to cooking, extraction of essential oils and flavoring material from plants degradation of vegetable tissues and the extraction of green tea components, modifying food materials such as vegetables, soybeans and rice, to increased the yield of the nutrients (www.govnews.org/mhonarc/gov/usfed/nara/fed-register/rules/msg00487.html). New products were obtained by treatment of cellulosic materials with cellulases to produce carbohydrates, which can be used as food or for alcohol fermentation, or for industrial chemicals and beverages (Winkelmann, 1992). Cellulose microfibril fragments can be used as noncaloric food additives. Hyperabsorbent cellulose fibers from fragmented cellulose microfibrils are used in biomedical and household absorbent material (Winkelmann, 1992). Cellulases are used in the pulp paper industry (Yinbo et al., 1996). They are widely applied in textile processing to improve fabric appearance by reducing fuzz, piling, and enhancing the softness, luster and color brightening of cotton fabrics (Ohmiya et al.,1997). Cellulases are also widely used as digestion aids (www.amermed.com/enzymes.html) and as detergents (Ito, 1997; Ozaki et al., 1995; Murata et al., 1991, 1993).

Fungi are the main cellulase producing microorganisms, even though a few bacteria and actionomycets have also been reported to produce cellulases (Miyamoto, 1997; Varma et al., 1994). Cellulases from fungi and bacteria have been studied extensively, but little attention has been given to cellulase from yeasts. To our knowledge this is the first report about the cellulases from yeast *Trichosporon* sp. and it is the second about yeast cellulases (Oikawa et al., 1998). Yeast cellulases proved to work at a broad range of both pH and temperature. Also, they have a reasonable degree of pH and thermal stability (Oikawa et al., 1998). These properties make them suitable for biotechnological processes. All these advantages and privileges stimulated our interest to conduct an extensive study on yeast cellulases from different perspectives.

For objecting this aim, the work was focused on the following main points:

1) Isolation and screening of cellulase producing yeasts.
2) Identification of a cellulase producing yeast strain.
3) Optimization of cellulase production by yeast isolate.
4) Purification of the produced cellulases.
5) Characterization of the purified enzyme.

2 MATERIALS

2.1 Organism:

The yeast strain PAG1 was isolated from the gut contents of the bug *Pyrrhocoris apterus* on GYP agar medium as described by Prillinger et al., (1996) at the Institute of Applied Microbiology and Mycology, University of Ulm, Germany.

2.2 Chemicals

Chemical	Company
Agar	Hartge-Marcor
Acrylamide/bisacrylamide (40%)	Sigma
Acetone	Roth
Acetic acid	Roth
Acetonitrile	Merck
Agarose NA	Roth
$AgNO_3$	Sigma
Ammonium sulfate	Roth
Ammonium persulfate	Merck
Avicel	Fluka
$BaCl_2$	Fluka
Boric acid	Roth
Bovine serum albumine	Merck
Bromophenol blue	Merck

$CaCl_2$	Fluka
Cellulose powder	Fluka
Carboxymethylcellulose (CMC)	Sigma
Cellobiose	Roth
Serva Violet 17 Staining Kit	Serva
Coomassie Brillant blue G250	Merck
Coomassie Brillant blue R 250	Merck
Congo red	Merck
Cysteine hydrochloride	Serva
dNTP	Fermentas
3,5-Dinitrosalicylic acid	Sigma
Dimethylsulfoxide	Serva
Ectoin	Biorad
EDTA	Roth
Ethylenglycol	Roth
Ethanol	Roth
$FeSO_4$	Merck
Gentiobiose	Sigma
Glucose	Merck
Glutathione	Merck
Glycerin	Merck
HCl	Roth
$HgCl_2$	Fluka
InstaGene-DNA-Purification-Matrix	Biorad

IEF marker protein (3-10)	Serva
Iodoacetate	Serva
KCl	Roth
KH_2PO_4	Roth
Lactose	Roth
LiCl	Roth
Maltose	Sigma
Methanol	Roth
Melizitose	Sigma
Mercaptoethanol	Merck
$MnCl_2$	Merck
$MgCl_2$	Merck
NaCl	Roth
NaOH	Roth
P-Nitrophenyl-ß-glucane derivatives	Sigma
Na_2SO_3	Merck
Peptone	Difco
$Pb(CH_3COO^-)_2$	Merck
Pluronic F68	Sigma
Potassium sodium tartrate	Merck
Primer IST1, IST4	MWG-Biotech AG
Proline	Sigma
Pyroglutamate	Biorad
Phenol	Roth

Raffinose	Sigma
Salicin	Sigma
Serva Violet 17 staining kit	Serva
Silicone antifoam	Serva
Sucrose	Roth
SDS	Biorad
Sodium azide	Sigma
Taq DNA-Polymerase	Applied Biosystems
TEMED	Serva
Trichloroacetic acid	Roth
Triton X-100	Merck
Tris	Roth
Tween 40 and 80	Merck
Yeast extract	Hartge-Marcor
Urea	Fluka
Xylan	Sigma

All chemical were of analytical grade.

2.3 Media

All media were autoclaved at 121 ^{o}C for 15 min except Bacto Yeast Nitrogen Base **(**DIFCO) was sterilized by membrane filter.

2.3.1 CMC agar medium

Per litre

CMC	5 g
Glucose	20 g
Yeast extract	5 g
Agar	15 g

2.3.2 Maintenance medium

Stock cultures of yeast strain were maintained at 4 ^{o}C and subcultured every 4 weeks on GYP agar medium.

Per litre

Glucose	20 g
Peptone	10 g
Yeast extract	5 g
Agar	15 g

2.3.3 GYP medium

For preparing overnight inoculums

Per litre

Glucose	20 g
Peptone	10 g
Yeast extract	5 g

2.3.4 Basal salt medium

(Lingens and Oltmanns, 1964)

Per litre (50 mM sodium phosphate buffer pH 7)

Yeast extract	5.0 g
$(NH_4)_2SO_4$	1.2 g
NaCl	0.5 g
$MgCl_2.6H_20$	0.7 g
KH_2PO_4	1.0 g
$FeCl_3.6H_2O$	0.05 g
$CaCl_2$	0.1 g
Glucose	10 g

The pH was adjusted to 7.0 before sterilization; glucose was sterilized separately and added to the medium before inoculation.

2.3.5 Bacto Yeast Nitrogen Base

Ten g of dehydrated Bacto Yeast Nitrogen Base W/O Amino Acids (DIFCO) medium with 10 g lactose were solved in 1 l of 50 mM sodium phosphate buffer pH 7.0 and filter sterilized. This medium was used as a production medium when the purification was objected, since it contains no protein, the cellulase purification may be easier.

2.4 Solutions and reagents

2.4.1 Protein stain solutions

- Stock solution I: 1 g SERVA violet 17 in 500 ml H_2O.

- Stock solution II: 233 ml H_3PO_4 (86 %) in 1000 ml H_2O.

- Destain solution: 35 ml H_3PO_4 (86 %) in 1000 ml H_2O.

- Fixation solution: Trichloroacetic acid (20 %) stored at room temperature.

2.4.2 CMC agarose

CMC agarose was prepared by dissolving 1% CMC in 50 mM sodium phosphate buffer pH 7. Equal volume of agarose (1%) was added and the mixture was boiled in microwave oven for 3 min, and then cooled to 40 ^{o}C.

2.4.3 Sample buffer

Three hundred µl water, 300 µl of 1 % SDS, 125 µl stacking gel buffer, 200 µl 50 % glycerol and 50 µl 0.05 % 2-mercaptoethanol.

2.4.4 Electrophoresis buffer

Three g Tris, 14.4 g Glycin, 10 ml SDS (10 %) in 1000 ml H_2O.

2.4.5 SDS gel stain solution

2.5 g Coomassie brillant blue R-250 in a mixture (1l) of water /methanol/acetic acid, (5:1:5)

2.4.6 DNS-reagent

(Miller, 1959)

DNS	10 g
Phenol	2 g
Na_2SO_3	0.5 g
NaOH	20 g
Potassium sodium tartrate	400 g

Components were dissolved in 1 liter of distilled water under stirring and heating at ca 50 ^{o}C and stored in brown glass bottles at room temperature.

2.4.7 Bradford reagent

(Bradford, 1976)

Hundred mg coomassie brillant blue G250 was dissolved in 50 ml of 96% ethanol and then, 100 ml of 85% phosphoric acid was added to the solution. The solution was brought to 1 l with water, filtered through a Whatman No 1 paper and stored in brown glass bottle at 4 ^{o}C.

2.4.8 TBE buffer

(Stock solution)

One hundred eight g Tris, 55 g boric acid, 8.3 g EDTA in 1000 ml H_2O, pH 8.3 (diluted 1:10 before use).

2.5 Equipment:

- Agarose-minigel electrophoresis, (Biorad).
- Centrifuge, Eppendorf Model 5415D
- Centrifugal filter devices, (Microcon YM-10 KD, 0.5 ml, Amicon)
- Centrifuge, SS34 Rotor, GSA Rotor, Sorvall Rc-5B
- Fast protein liquid chromatography (FPLC); (Pharmacia Biotech). The system consisted of: Gradient programmer (GP-250); two-high precision pumps (P-500); 7-port injection valves; two V-8 port selection valves (Mv-8); single path monitor (UV-1); fraction collector (FRA C-100) and two-channel recorder (REC-482). The system was equipped with two columns, Mono Q HR 5/5 (Pharmacia Biotech) for anion exchange and Resource ISO (Pharmacia biotech) backed with isopropyl-Sepharose for hydrophobic interaction chromatography.
- High-performance liquid chromatography System (HPLC**),** (112 Beckman). Solvent delivery module was equipped with a 156 Beckman Refractive Index Detector; column Aminex HPX-42A, 300 mm x 7.8 mm and chromatographic data recorder (Shimadzu C-R6A Chromatopac).

- Gel electrophoresis apparatus, (Modell Z37, 302-8, Sigma).

- Isoelectric focusing (LKB, 2117 Multiphor II).

- Photometer, (Shimadzu UV 240).

- PCR-apparatus (Progene, Techn).

- Rotary shaker G10, (New Brunswick scientific Co., INC., Edison, N.J., USA).
- Thermomixer, (HLC, HTM130).

- Ultra-pure water system, (Milli-Q Plus 185, Millipore).

- Vortex VF2 (Janke&Kunkel Ika , Labortechnik).

- Water bath (Julabo SW-20C).

- Water purification system, (Milli-RO plus 30, Millipore).

3 METHODS

3.1 Identification of isolated yeast by PCR

3.1.1 DNA extraction

The isolated yeast strain was grown in GYP medium. One ml from the grown culture was centrifuged at 1300 rpm for 3 min in an Eppendorf centrifuge. The supernatant was discarded, and the yeast pellet was suspended in 1 ml sterilized water and centrifuged again as previously described; the pellet was suspended in 200 µl INstaGene-Matrix. The suspension was incubated at 56 ^{o}C for 20 min with shaking in a

thermomixer. The suspension was vortexed for 10 s, and incubated at 98 °C for 8 min with shaking and vortexed for 10 s. The suspension was centrifuged at 1300 rpm for 3 min. The supernatant was transferred to 1.5 ml tubes and stored at -20 °C

3.1.2 PCR amplification

(Messner et al., 1994)

The PCR amplification reactions were performed in a total volume of 100 µl. Each reaction mixture contained the following solutions 1 µl DNA, 4 µl 10 pmol Alleu 3.1 primer (5?- TCTGCAGGTTCACCTAC- 3?); 4 µl of 10 pmol Alleu 5.1 primer (5?- TACCTGGTTGATCCTGCC-3?); 10 µl of 250 mM dNTP's; 10 µl PCR buffer, 3.5 µl 25 mM $MgCl_2$ and 0.5 µl Taq polymerase and water was added up to 100 µl.

The PCR-appratus was programmed as follows: 5 min denaturation at 94 °C, followed by 35 cycles that consisted of 60 s at 94 °C, 80 s at 50 °C and 150 s at 72 °C, and a final 10 min extension at 72 °C. The primer Alleu 5.1(5?- TACCTGGTTGATCCTGCC-3?) was used as a sequence primer. For more information, 18S rDNA was amplified by PCR using the following primers: Alleu 3.1 (5?- TCTGCAGGTTCACCTAC- 3?) and Oxy 5.1 (5?- CCAGCAGC(T/C)GCGGTAATT-3?). PCR was performed using the following program: 5 min denaturation at 95 °C, followed by 35 cycles that consisted of 30 s at 95 °C, 30 s at 50 °C, 120 s at 72 °C and a final 10 min extension at 72 °C. The primer Alleu 3.1 (5?- TCTGCAGGTTCACCTAC- 3?) was used as a sequence primer. Also IST1 primer (5'-TCC GTA GGT GAA CCT GCG G-3') and IST4 primer (5'-TCC TCC GCT TAT TGA TAT

GC-3') were used for ITS region analysis. The PCR-appratus was programmed as follows: 5 min denaturation at 95 ^{o}C, followed by 35 cycles that consisted of 90 s at 95 ^{o}C, 45 s at 50 ^{o}C and 2 min at 72 ^{o}C, and a final 5 min extension at 72 ^{o}C.

The products of the PCR amplification were analyzed by agarose gel electrophoresis (1%). Five µl PCR products were mixed with 1 µl of gel loading buffer. The mixture was loaded on agarose gels (1% w/v) in TBE buffer (0.045 M Tris, 0.045 M boric acid, 0.001 M EDTA, pH 8). The separation was carried out at 90 V for 40 min and the gel was submerged in 8 mM ethidium bromide solution for 20 min.

The resulting DNA patterns were examined with UV light and photographed**.**

3.1.3 DNA-sequencing

The DNA was sequenced by the Genterprise Company (Mainz).

3.2. Cellulases screening test

As described by Farkas et al., (1985) screening test was carried out by pouring a suitable amount of CMC agar medium into petri dish. The medium was allowed to solidify. The yeast cells were streaked onto the agar. After incubation, the plate was flooded with a Congo red solution (0.2% w/v) for 15 min, and then destained with 1 M sodium chloride by washing the plate with destaining solution several times. Unstained areas indicated hydrolysis of cellulose.

3.3 Determination of cellulolytic activities

The cellulo lytic activities were determined by the colorimetric method of Miller (1959) using the DNS-reagent. This method based on the determination of the color developed after the reaction between the reducing sugars liberated from cellulase and DNS-reagent

Procedure:

Two ml of growth medium were transferred to 2 ml plastic microtubes, and centrifuged in an Eppendorf centrifuge at 13000 rpm for 5 min. The supernatants were transferred carefully to vials and stored on ice until required for activity assaying. From the culture supernatant, 0.25 ml were incubated with 0.9 ml of a CMC solution (1 %; w/v) in 50 mM sodium phosphate buffer (pH 7.0) and 0.25 ml distilled water at 40 ^{o}C for 30 min. Blanks were prepared in the same way and placed in ice instead of incubation. The determination was carried out in duplicate. After incubation, the enzyme activity was stopped by adding 1.5 ml DNS-reagent; tubes were placed in a boiling water bath for 15 min, cooled down to room temperature. The O.D. of the samples was immediately measured at 575 nm. One enzyme unit was defined as 1 µmol of glucose equivalents released per min.

3.4 Isoelectric focusing (IEF)

Analytical IEF was performed by using a SERVALYT PRECOTES-Gel R 3-10, (150µm, 125mm x 125mm) and IEF marker protein 3-10. The enzyme samples were desalted and adjusted to concentrations of 0.1-5 mg/ml.

Five µl of the sample and of the marker protein solution were loaded onto the gel. Electrophoresis was carried out at 2000 V, 3 mA and 6 W for 2.5 h at 5 ^{o}C.

• Fixation: Gel was transferred to 200 ml of a trichloroacetic acid solution (20 %; w/v) for 20 min followed by rinsing with water for 1 min.

• Staining: Gel was stained with a freshly prepared mixture of stock solutions I and II (200 ml; 1:1; v/v).

• Destaining: Gel was destained in 300 ml destaining solution 2 - 3 times, 10 min each, followed by rinsing with water twice for 2 min.

• Drying: Gel was left overnight for drying at room temperature.

To detect cellulase activity, samples were loaded onto the gel in duplicate. After electrophoresis the gel was cut longitudinally into two pieces. One piece was direct stained with SERVA violet 17, and other was used for testing the activity. The gel was equilibrated in 50 mM sodium phosphate buffer (pH 7), covered with a layer of CMC-agarose and incubated at 37 ^{o}C for 6 h. Finally the gel was stained for 30 min in 0.1% Congo red and destained overnight in 5 mM NaOH solution containing 1 M NaCl.

3.5 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis under denaturing and non-denaturing conditions was carried out by the method of Laemmli (1970)

Required solutions:

1) Separating gel buffer: 1 M Tris/ HCl pH 8.8.

2) Stacking gel buffer: 1 M Tris/HCl pH 6.8.

3) CMC (1 %) solution in water.

4) Acrylamide / bisacrylamide (40 %)

5) TEMED.

6) Sodium dodecyl sulfate (SDS; 10 %)

7) Ammonium persulfate (APS; 10 %)

8) Sample buffer (2.4.3)

• Gel composition (polyacrylamide 10 %)

Solution	Gel for cellulase determination	Gel for protein determination
water	0.75 ml	1.95 ml
Separating gel buffer	2.26 ml	2.26 ml
CMC (1 %)	1.2 ml	0.0 ml
Acrylamide (40 %)	1.5 ml	1.5 ml
SDS (10 %)	0.0 µl	60 µl
APS (10 %)	48 µl	48 µl
TEMED	5 µl	5 µl

• Stacking gel

Water	3.7 ml
Stacking gel buffer	625 µl
Acrylamide (40 %)	625 µl
SDS (10 %)	50 µl
TEMED	5 µl
APS (10 %)	50 µl

Procedure: The separating gel solutions were combined, degassed and poured between two glass plates, covered with a layer of water and allowed to polymerize for 30 min. The water was removed, and stacking gel solutions were degassed and poured over the separating gel. A comb was inserted into the gel and the gel was allowed to polymerize. Twelve µl of the sample were mixed with 4 µl of sample buffer and introduced into the wells. The gel was run in the presence of electrophoresis buffer at 50 V until the bromophenol blue front migrated into the resolving gel. The voltage was increased to 150 V, until the gel dye reached the bottom of the resolving gel. The gel was stained for 30 min in a solution of coomassie brillant blue R-250(0.25 %) and destained overnight in acetic acid solutions (7 %).

The native gel with CMC was carried out according to the same procedures but the samples were applied in replicates. After running the gel was cut longitudinally into 2 pieces. One was stained with coomassie stain and the other was used for the determination of the cellulase activity.

After fixation for 1 h the gel was twicely equilibrated in 50 mM sodium phosphate buffer for 15 min each. Gel was incubated at 37 ^{o}C for 6 h, stained for 30 min in 0.1 % Congo red and destained overnight in 1 M NaCl.

3.6 Protein determination

The measurement of protein was done as described by Bradford (1976), using BSA as standard. Hundred µl of the sample was mixed with 2 ml Coomassie brillant blue G250 reagent and kept at room temperature for 10 min. The absorbance at 595 nm was measured on a spectrophotometer, (Shimadzu UV 240) against a blank. The calibration curve was obtained between a BSA concentration range of 10 - 500 µg/ml.

3.7 Preparation of phosphoric acid-swollen avicel

(Wood, 1971)

Avicel (10 g) was suspended in concentrated phosphoric acid (88 % w/v) and kept with occasional stirring for 1 h at 1 ^{o}C. The mixture was incubated in a ice-cold water (4 l) for 30 min. The swollen avicel was washed several times with cold water. After washing with a $NaHCO_3$ solution (1 %; w/v), the suspension of swollen avicel was dialyzed at 1 ^{o}C against water. After a 60 s treatment in Blender, water was added to the suspension until to a final concentration of 1 mg/ml. Sodium azide was added to the suspension (final concentration 5 mM) and stored at 1 ^{o}C.

3.8 Preparing dialysis tubing

In order to remove chemical impurities, the dialysis tubings (Visking type 36/32, Roth) were boiled for 30 min in 10 mM sodium bicarbonate containing 1 mM EDTA. The tubing was washed extensively in distilled water and stored at 4 ^{o}C in 1 mM EDTA to prevent microbial contamination.

3.9 Optimization of culture condition

3.9.1 Inoculum preparation

The yeast cells were collected from the maintenance cultures (slant agar) and suspended in 5 ml sterilized 50 mM sodium phosphate buffer pH 7.0 and centrifuged at 10000 rpm for 5 min in an Eppendorf centrifuge, Model 5415D.

The supernatant was decanted and the pellet again suspended in the same buffer and centrifuged. The obtained pellet was suspended in 2 ml of the same buffer and transferred into a 100 ml conical flask containing 18 ml of cultivation medium and incubated for 24 h at 30 ^{o}C on a rotary shaker at 200 rpm.

3.9.2 Experimental media

The basal salt medium was used to investigate the effects of nutritional and environmental factors on yeast growth and cellulase excretion. The medium was modified according to the conditions and factors, which were studied. Twenty milliters of a test medium was transferred into 100 ml Erlenmeyer flasks, plugged and autoclaved. The sterilized media were inoculated with the strain and incubated at the test temperature (5 ^{o}C - 60 ^{o}C) on a rotary

shaker at appropriate rpm (0 - 400) for a certain time (0 - 72 h), according to the goal of test. After the incubation, samples were taken for cellulase determination.

3.9.3 Incubation temperature

To find the optimum temperature for growth and cellulase production, the strain was cultivated in basal salt medium containing 1% carboxymethylcellulose (CMC) instead of glucose. Incubation was carried out at different temperatures (5 ^{o}C to 60 ^{o}C) for 18 h under steady state.

3.9.4 Selection the of the carbon source

To select the best polymeric carbon source for the induction of cellulases, different types of cellulose i.e. avicel; cellulose powder and CMC were separately added as a sole carbon source to the basal media in concentration of 1% (w/v) instead of glucose. The media were inoculated and incubated at 30 ^{o}C for 18 h under shaking (100 rpm)

3.9.5 CMC concentration

CMC with medium or high viscosity was added to the basal salt media as a carbon source at several concentrations (0.25 % to 1.5 %; w/v). Media were inoculated and incubated at 30 ^{o}C for 18 h with shaking at 100 rpm.

3.9.6 Selection of the nitrogen source

To find the best nitrogen source, various nitrogen compounds i.e. meat extract, peptone, urea, $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$ were separately added in

a concentration of 1 % (w/v) to media containing CMC (0.5%; w/v) and yeast extract (YE; 0.5 % w/v). The media were adjusted to pH 7, inoculated and incubated at 30 ^{o}C for 18 h with shaking at 100 rpm.

3.9.7 Peptone concentration

Peptone was added as nitrogen source to the media, which contained CMC (0.5 %) and YE (0.5 %) in concentrations ranging from 0.1 to 2.0 (%; w/v). Media were adjusted to pH 7, inoculated and incubated at 30 ^{o}C for 18 h under shaking 100 rpm.

3.9.8 Medium pH value

The effect of the pH value was investigated by cultivation the isolate in media containing peptone (0.5 %; w/v), CMC, (0.5 %; w/v) and YE (0.5 %; w/v). Media were adjusted to different pH values from 2 to 12 with NaOH (0.4 M) or HCl (0.4 M). Media were inoculated and incubated at 30 ^{o}C for 18 h under shaking 100 rpm.

3.9. 9 Surfactants

Surfactants e.g pluronic F68, silicone antifoam, Tween 40 and Tween 80 were added to the cultivation media which contained CMC (0.5 %; w/v), peptone (0.5 %; w/v) YE (0.5 %; w/v) as a concentration of 0.1 %. Controls were prepared in the same way without surfactants. The media were inoculated and incubated at 30 ^{o}C for 18 h with shaking at 100 rpm.

3.9.10 Tween 80 concentration

Tween 80 was added to the culture media at concentrations that ranged from 0.1 % to 2 % (v/v).

3.9.11 Induction

To induce cellulase biosynthesis different saccharids e.g cellobiose , maltose, salicin, raffinose, lactose, sucrose and polymers e.g. xylan were separately added at a concentration of 1% (w/v) to the growth media which contained CMC (0.5 %; w/v) peptone (0.5%; w/v) YE (0.5 %; w/v), Tween 80 (0.5%; v/v). Controls were prepared in the same way, except that no inducer was added. The media were inoculated and incubated at 30 ^{o}C for 18 h under shaking 100 rpm.

3.9.12 Lactose concentration

Various concentrations of lactose ranging (0.05 to 1.5 %; w/v) were added to the culture media to find the optimal inducer concentration. Controls were prepared in the same way, without inducer was omitted.

3.9.13 Culture agitation

Erlenmeyer flasks (250 ml) containing media composed of CMC (0.5 %; w/v), peptone (0.5 %; w/v), YE, (0.5 %; w/v), Tween 80 (0.5 %; v/v) and lactose (1 %; w/v) were inoculated and placed onto a rotary shaker at different rpm i.e. (0, 100, 200, 300, 400 and 500 rpm) for 18 h at 30 ^{o}C.

3.9.14 Cultivation time

Erlenmeyer flasks (250 ml) containing 50 ml of media composed of CMC (0.5 %; w/v), peptone (0.5 %; w/v), YE, (0.5 % w/v), Tween 80 (0.5 % v/v) and lactose (1%; w/v) were inoculated, incubated with shaking at 200 rpm at 30 ^{o}C. Samples were withdrawn at different times to measure the cellulolytic activity.

3.10 Purification of cellulase

3.10.1 Preparation of crude enzyme

All procedures were carried out at 4 ^{o}C. Yeast culture was centrifuged at 10000 x g for 20 min to remove the cells. For partial purification, solid ammonium sulfate (30 % saturation; 176 g/l) was added to the supernatant. The mixture was centrifuged at 10000 x g for 20 min. The sediment was discarded. Solid ammonium sulfate was added under stirring to a final saturation of 80 % (351g/l) saturation. The suspension was stirred for 1 h and kept overnight**.** The precipitate was collected by centrifugation at 38000 x g for 30 min. The pellet was dissolved in 20 mM Tris/HCl, pH 7.6 which contained NaN_3 (0.32 g/l) to prevent microbial growth.

For desalting, dialysis was carried out against 20 mM Tris/HCl buffer pH 7.6 overnight at 4 ^{o}C under stirring. Finally, the desalted protein solution was centrifuged at 13000 rpm to remove any undissolved material for 5 min (Eppendorf centrifuge Model 5415D).

3.10.2 Chromatography

All purification steps were performed at room temperature, all solutions used for chromatography and enzyme tests were prepared by dissolving compounds in water (Millipore ultra-pure water system, Milli-Q Plus 185). In addition the solutions were filtered through a 0.45 µm filter and degassed by stirring for 30 min under vacuum. The purity of different enzyme preparations was tested by SDS-gel electrophoresis.

Enzyme purification was performed with fast protein liquid chromatography (FPLC, Pharmacia Biotech) at room temperature. The system was equipped with two columns; Mono Q HR 5/5 for anion-exchange chromatography and Recourse ISO for hydrophobic interaction chromatography.

3.10.2.1 Separation by anion-exchange

The Mono Q column was equilibrated with 20 mM Tris / HCl buffer at pH 7.6. Then, the sample was applied. Elution was performed with a linear gradient of 2 M NaCl in 20 mM Tris/HCl buffer at a flow rate of 1 ml/min. Fractions (1 ml) were collected and assayed for enzyme activity. The activity was observed in fractions 11 - 15 as 2 major peaks (PII, PIII, Fig. 14 14). The fractions were pooled, desalted, concentrated and buffer exchanged by 20 mM sodium phosphate buffer pH 7 by using Amicon centrifugal filters (Microcon YM-10).

3.10.2.2 Fractionation by hydrophobic interaction (HIC)

The enzyme solution of peak II (Fig. 15) was adjusted to 1.5 M ammonium sulfate by adding solid ammonium sulfate and loaded onto the Resourse ISO column, which was then equilibrated with 20 mM sodium phosphate buffer pH 7 containing 2 M ammonium sulfate. Elution was performed with the same buffer but without ammonium sulfate at a flow rate of 1 ml/min. Fractions (1 ml) were collected and assayed for enzyme activity. Fractions with enzyme activity were combined, desalted and concentrated. The buffer was exchanged with 20 mM Tris / HCl buffer, pH 8, by using Amicon centrifugal filters (Microcon YM-10).

3.10.2.3 Rechromatography

The enzyme preparation of peak II (Fig. 16) was separated with a Pharmacia Mono Q HR 5/5 column.

The column was equilibrated with 20 mM Tris/HCl buffer, pH 8. Elution was carried out with a linear gradient of 2M NaCl in 20 mM Tris /HCl buffer pH 8, at a flow rate of 1 ml/min. Active fractions (peak I, Fig.16) were combined, desalted and used as pure enzyme preparation (cellulase I) for further characterization.

3.11 Characterization of cellulase

All determinations were performed in duplicates and measured against blank samples.

3.11.1 pH dependence

The pH value of the purified enzyme solution was adjusted between 2 and 11 by using an Amicon centrifugal filter tube (0.5 ml). The substrate was solved in the same buffer as the enzyme. The enzyme (0.1 units) was incubated with 800 µl of substrate (CMC; 1 %; w/v) for 30 min. Afterwards the enzyme activity was estimated with the DNS method (Miller, 1959). The following buffers (50 mM) were used: (a) glycin / HCl, pH 2 – 3, (b) citrate pH 4 – 5, (c) sodium phosphate pH 6 – 7, (b) Tris / HCl pH 8 and glycin / NaOH pH 9 - 11.

3.11.2 pH stability

The enzyme (0.1 units) was incubated in different buffer at 4 ^{o}C for 3h (3.12.1). The pH was adjusted to 5. The remaining activity was determined with the DNS standard method (Miller, 1959).

3.11.3 Temperature optimum

The purified enzyme (0.1 units) was incubated with the substrate (1 % CMC) in citrate buffer (pH 5) at various temperatures from 4 ^{o}C to 60 ^{o}C for 30 min. The reducing sugars were determined with DNS method.

3.11.4 Thermal stability

The purified enzyme (0.1 units) was incubated in 50 mM citrate buffer at different temperatures ranging from 4 ^{o}C to 70 ^{o}C for 30 min. Then the remaining activities were determined with the DNS standard method.

3.11.5 Chemical compounds

The enzyme activities were measured with DNS method in the presence of various compounds: pyroglutamate, proline, ectoin, hydroxyectoin, EDTA, glycerol, 2-mercaptoethanol, cysteine hydrochloride, tween 40, Na_3N, tween 80, pluronicF68, triton X-100, silicone antifoam, SDS, iodoacetate and glutathione (0.1%, final concentration).

3.11.6 Metal ions

The enzyme activities were determined with the DNS method in the presence of different metal ions (1 mM, final concentration):
$CdCl_2$, $BaCl_2$, $CaCl_2$, $FeCl_3$, KCl, $MnCl_2$, $MgCl_2$, LiCl, NaCl, $Pb(CH_3COO^-)_2$, $HgCl_2$, and $Ag\,NO_3$.

3.11.7 Organic solvents

The influence of different organic solvents (20 %, v/v; final concentration) methanol, ethanol, ethylenglycol, toluene, dimethylsulfoxide, acetone and acetonitrile on the cellulase activity was studied using (1 %, CMC) as substrate.

3.11.8 Inhibition by oligosaccharides

In order to test the influence of oligosaccharides on the enzyme activity, various reducing and nonreducing oligosaccharides such as cellobiose, lactose, sucrose, gentiobiose, raffinose, melizitose and maltose

were added separately to the incubation mixtures with a concentration ranging from 0.6 - 2.8 mM.

3.11.9 Substrate concentration

In order to determine the kinetic constants of the purified cellulase (I) (K_m and V_{max} values), CMC was used as substrate with varying concentrations ranging from 0.1 % - 2.2 % under optimal conditions (30 min, pH 5, 40 ^oC). The apparent K_m and V_{max} values were calculated by using enzyme program (http://www.uni-mainz.de/FB/Biologie/Mikrobiologie/download/download.htm).

3.11.10 Activity towards different substrate

In order to study the specific activity of the purified isolated enzyme, insoluble cellulosic substrates namely, sigmacell (microcrystalline cellulose), xylan, cellulose powder, H_3PO_4-swollen avicel and soluble substrate i.e. carboxymethylcellulose (CMC), cellobiose, raffinose, melizitose, sucrose and p-nitrophenyl derivatives such as : p-nitrophenyl-ß-D-glucuronide, 2-nitrophenyl-ß-D-galactopyranoside, p-nitrophenyl-ß-xylopyranoside, p-nitrophenyl-ß-D-glucopyranoside and p-nitrophenyl-ß-xyloside were suspended or solved in 50 mM citrate buffer pH 5 in a concentration of 1 % (w/v). Insoluble substrates were treated by ultra sonication for 8 min. The activities were measured by incubating 0.1 mg of the purified enzyme with 0.9 ml of each substrate solution or suspension at 40 ^oC for 30 min (soluble substrate) or 48 h (insoluble substrate). The reducing sugars were determined by the DNS method.

In case of p-nitrophenyl derivatives, reactions were stopped by addition of 2 ml of 1 M $NaCO_3$ and p-nitrophenol release was monitored at E_{400}. One enzyme unit corresponded to 1 µmol of p-nitrophenol released per min.

3.11.11 Saccharification of cellulosic materials

An attempt was carried out to evaluate the ability of the cellulase system (cellulase I purified enzyme; ß-glucosidase crude enzyme) of the used strain for converting cellulosic materials to glucose monomers (Beldman et al., 1985; modified)

Substrates:

(a) H_3PO_4-swollen avicel (1 %; w/v) in 50 mM sodium citrate buffer pH 5.

(b) CMC (1 %; w/v) in the same buffer.

Enzyme preparation:

(c) purified cellulase (0.1 units).

(d) ß-glucosidase (0.1 units).

Incubation mixture:

(1) a + c

(2) b +c

(3) a + c + d

(4) b + c + d

The reaction mixture was incubated at 40 ^oC for 16 h, boiled for 5 min in water bath, and centrifuged at 1000 x g for 3 min. The supernatants were filtered through syringe filter (0.45 µm Minisart, Sartorius) and analyzed by

HPLC equipped with an Aminex column HPX-42A; (300 mm x 7.8 mm). Twenty µl of each sample was applied onto the column and eluted with deionized water at a flow rate of 0.6 ml/min. Glucose, cellobiose, cellotriose, cellotetraose and cellopentaose were used as standards.

4 RESULTS

4.1 Morphology of the yeast isolate

The isolated yeast strain showed formation of filamentous hyphae and propagated with budding (Fig. 5). Its optimal growth was observed at 30 °C

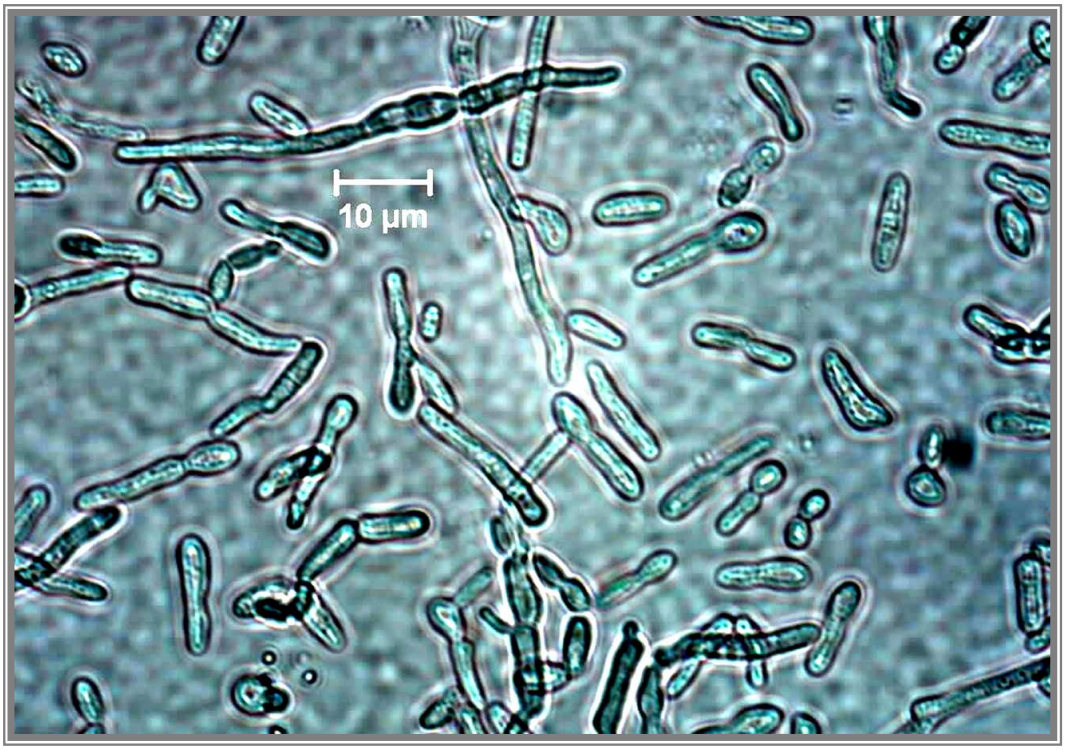

Fig. 5. Micrograph of the isolated yeast strain PAG1 after two days of cultivation in a modified yeast nitrogen base medium (2.3.5).

The isolated yeast formed rugose white-yellow colonies on GYP agar medium (Fig. 6).

Fig. 6. Morphology of isolated yeast strain PAG1. (medium 2.3.2).

4.2 Identification of yeast isolate

For molecular identification of the isolated yeast, strain PAG1. Amplification of isolated yeast DNA with general primers (Alleu 5.1/ Alleu 3.1) for the 18S rDNA gave a single band of about 750 bp (Fig. 7). The

obtained fragment was sequenced with Alleu 5.1. The sequences were analyzed with the BLAST program (EMBEL Gen Bank). The isolated strain showed 100 % sequence identity with *Trichosporon japonicum*, *T. asahii, T. aquatile, T. faecale*, *T. coremiiforme*, *T. aquatile* and *T. asteroides*

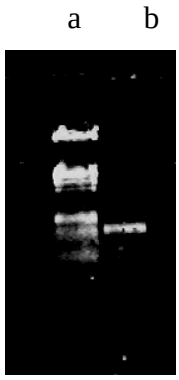

Fig. 7. Amplification products with primers (Alleu 5.1/ Alleu 3.1).

(Lane a: molecular marker; Lane b: investigated strain)

To obtain further information about the phylogenetic relationship of the isolated yeast. PCR was carried out with additional 18S rDNA primers (Oxy 5.1/ Alleu 3.1). A single fragment of 800 bp (Fig.8) was produced by amplification of isolated yeast DNA with primers Oxy 5.1/ Alleu 3.1. The produced band was sequenced using Alleu 3.1 or Oxy 5.1 as sequence primer. The sequence analysis also revealed a 100 % identity with the above mentioned species.

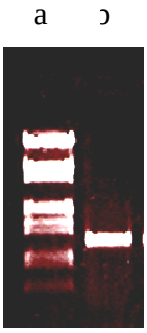

Fig. 8. Agarose gel electrophoresis of PCR products of isolated yeast with primers (Oxy 5.1/ Alleu 3.1).

Lane a: molecular size marker; lane b: studied strain

Also the analysis of ITS region with ITS_1/ITS_4 primer led to the same result (100 % identity). The complete identification of the isolated yeast and its assignment to one species (cf. Fig.9) requires physiological and morphological tests in addition to molecular methods of DNA analysis.

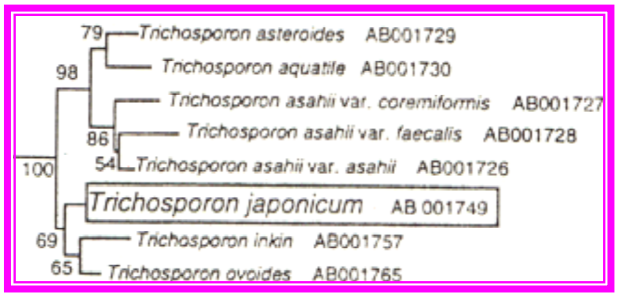

Fig. 9. Selected region of a Neighbour-joining tree (Sugita and Nakase, 1998) of species related to the investigated strain PAG1.

4.3 Cellulolytic ability of yeast isolate

The isolated yeast strain was cultivated on CMC agar plates for 5 days, to test its cellulolytic ability. After staining with Congo red, a clear zone around the colony was observed, this indicated cellulase production (Fig. 10).

Fig. 10. Activity staining of the isolated yeast PAG1 on agar plate.
The yeast was grown on CMC–agar medium (2.3.1).
The plate was flooded with solution of 0.1 % (w⁄v) congo red
for 30 min and destained with 1 M NaCl for 1 h.)

4.4 Factors affecting cellulase production

3.4.1 Effect of incubation temperature

The yeast strain was grown in the cultivation medium (3.9.2) at various temperatures (5 °C to 60 °C) to define the optimal temperature for growth and cellulase production. The results were summarized in Table 1. The yeast was able to grow and accumulate the cellulase in a broad range of incubation temperatures from 5 °C to 50 °C for 18 h. No growth was observed at 60°C. The optimum growth and cellulase production was found at 30 °C.

Table 1. Effect of incubation temperature on cellulase production.

Incubation temperature (°C)	Biomass dry wt* (mg/ml)	Cellulase activity (U/ml)
5	7.88	0.021
10	8.88	0.022
15	8.90	0.025
20	12.00	0.042
25	12.25	0.042
30	12.88	0.057
35	12.86	0.053
40	7.75	0.043
45	6.78	0.017
50	6.26	0.013
60	4.45	0.000

*** Dry weight of noculums biomass was (6 mg/ml)**

4.4.2 Effect of carbon source on cellulase production

Different cellulose types such as CMC, microcrystalline cellulose (avicel), and amorphous cellulose were added separately to growth medium (3.9.4) as sole carbon source. They resulted in cellulase production (U/ml) of 0.0886, 0.0188, and 0.0136 respectively. The maximum cellulase yield was obtained with CMC while the minimum production was found in the presence of cellulose powder.

4.4.3 Effect of CMC concentration on cellulase production

Various concentrations (0.25, 0.5, 1.0, and 1.5 %; w/v) of medium and high viscosity CMC were added to the cultivation medium (3.9.5). The cellulase activity was calculated in each case separately as presented in Table 2. The best results were obtained with 0.5 % of medium viscosity of CMC.

4.4.4 Effect of various nitrogen sources

Organic (meat extract, peptone, urea) and inorganic $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$, compounds were added to the culture media to measure their effect on cellulase production. The results of this study were presented in Table 3. They showed that all examined compounds except urea stimulated the growth and cellulase production. The organic compounds stimulated higher cellulase yields compared with inorganic compounds. The highest production was obtained with peptone while the lowest was resulted by using $(NH_4)_2HPO_4$.

Table 2. Effect of CMC-M.V and CMC-HV concentrations on cellulase production.

CMC concentration (w/v; %)	Biomass dry wt [mg/ml]		Cellulase activity (U/ml)	
	M.V*	H.V**	M.V*	H.V**
0.25	15.62	15.00	0.0763	0.067
0.5	15.31	15.60	0.0830	0.089
1.0	15.31	15.67	0.047	0.059
1.5	15.25	14.80	0.031	0.049

* Viscosity of 2 % aqueous solution at 25 ^{o}C: 400-800 centipoise (cps),** viscosity of 1 % aqueous solution at 25 ^{o}C: 1500-3000 cps

Meat extract induced the highest growth yield but lowest cellulase production in comparison to peptone.

Table 3. Effect of nitrogen compounds on cellulase production.

Nitrogen compounds	Biomass dry wt (mg/ml)	Cellulase activity (U/ml)
Meat extract	58.37	0.069
Peptone	52.66	0.078
Urea	6.67	0.000
$(NH_4)_2SO_4$	20.66	0.009
$(NH_4)_2HPO_4$	14.44	0.007

4.4.5 Effect of peptone concentration

The isolated yeast was grown in the presence of different peptone concentrations (0.1 to 2 %; w/v). The results are presented in Table 4. Generally, growth and cellulase production were obtained at all tested concentrations. The best results were obtained with 0.8 % w/v.

Table 4. Effect of peptone concentration on cellulase production.

Peptone concentration (% ; w/v)	Biomass dry wt (mg/ml)	Cellulase activity (U/ml)
0.1	47.25	0.077
0.3	50.14	0.091
0.5	52.88	0.095
0.8	56.59	0.099
1.0	56.66	0.081
1.3	57.65	0.078
1.5	56.44	0.066
2.0	59.37	0.066

4.4.6 Effect of medium pH value

The isolated yeast was cultured in medium at different pH values (2 to 12). Table 5 shows that the isolated strain was able to grow and produce cellulase in a wide pH range from 3 to 10. The highest cellulase yield was found at an initial pH value of 7.0

Table 5. Effect of growth medium-pH on cellulase production.

Adjusted pH	pH after sterilization	pH after cultivation	Biomass dry wt (mg/ml) before cultivation	Biomass dry wt (mg/ml) after cultivation	Cellulase activity (U/ml)
2	2.13	2.23	5.51	5.33	0.000
3	3.14	3.22	5.19	9.62	0.019
4	4.09	6.58	5.77	16.55	0.099
5	5.06	8.19	5.42	16.45	0.108
6	6.04	8.37	5.77	16.55	0.111
7	7.01	8.41	5.85	29.60	0.123
8	7.90	8.50	5.87	16.92	0.106
9	8.70	8.58	5.92	15.24	0.104
10	9.48	8.68	5.70	15.27	0.093
11	9.95	8.75	5.77	15.00	0.012
12	11.09	10.38	5.10	4.44	0.000

4.4.7 Effect of surfactants

The effect of surfactants was examined by supplementation of the growth medium with different surfactants (0.1 %; v/v). Data given in Table 6 show that all tested surfactants enhanced the cellulase production. The highest cellulase production was obtained with Tween 80 followed by Tween 40, then silicone antifoam and pluronic F68.

Table 6. Effect of surfactants on cellulase production.

Surfactants	pH after cultivation	Biomass dry wt (mg/ml)	Cellulase activity (U/ml)
None	8.26	49.65	0.071
Pluronic F68	8.38	53.98	0.101
Silicone antifoam	8.23	n.d*	0.101
Tween 40	7.72	n.d*	0.120
Tween 80	8.31	n.d*	0.152

* Not determined

4.4.8 Effect of Tween 80 concentration

Different concentrations of Tween 80 (0 - 2 %; v/v) were added to the culture medium to define the optimal concentration of Tween 80 needed to induce maximum cellulase yield.

The obtained results revealed that an increase of Tween 80 corresponded to an increase of cellulase production, as presented in Table 7. The highest cellulase yield was obtained in medium containing (0.5 %; v/v) Tween 80.

Table 7. Effect of Tween 80 concentration on the cellulase production.

Tween 80 (v/v; %)	Culture-pH after cultivation	Biomass dry wt (mg/ml) after cultivation	Cellulase activity (U/ml)
None	8.33	53.48	0.072
0.1	8.13	Nd*	0.120
0.25	8.07	Nd*	0.149
0.5	7.87	Nd*	0.160
1.0	7.51	Nd*	0.156
1.5	7.47	Nd*	0.160
2.0	7.2	Nd*	0.159

Nd* not determined

4.4.9 Induction of cellulase by different saccharides

The yeast strain was grown in medium (3.9.11) containing different saccharides to determine their effect on cellulase induction. Data in Table 8 indicated that no inducing effect was observed with salicin, raffinose, xylan and sucrose, whereas the cellulase yield was highly enhanced by addition of maltose, cellobiose and lactose. The optimal yield was found with lactose. Both cellobiose and maltose exhibited the same effect. The results indicated also that growth was correlated with the cellulase yield increase and

decrease except sucrose which stimulated high growth but low cellulase production.

Table 8. Induction of cellulase production by different saccharides.

Saccharides	pH after cultivation	Biomass dry wt (mg/ml) after cultivation	Cellullase activity (U/ml)
Control	8.15	53.39	0.081
Maltose	7.65	60.59	0.153
Salicin	8.05	52.91	0.085
Cellobiose	7.59	61.25	0.152
Raffinose	8.16	53.98	0.072
Lactose	7.67	57.55	0.180
Xylan	8.28	Nd	0.077
Sucrose	8.17	64.22	0.080

4.4.10 Induction of cellulase by lactose

Different concentrations of lactose (0 - 1.5 %; w/v) were added to the culture medium to define the optimum concentration, which induced the highest cellulase production. Table 9 shows that the cellulase production corresponded to the lactose concentration. The optimal concentration was found to be 1 % (w/v). On the other hand, growth was weakly influenced by lactose.

Table 9. Effect of lactose concentration on cellulase production.

Lactose (%; w/v)	pH after cultivation	Biomass dry wt (mg/ml) after cultivation	Cellulase activity (U/ml)
None	8.63	7.44	0.074
0.05	8.54	7.42	0.093
0.1	8.35	7.80	0.105
0.2	8.28	8.01	0.125
0.3	8.16	8.33	0.148
0.4	8.14	8.55	0.170
0.5	8.05	8.75	0.171
1.0	7.50	8.84	0.183
1.5	7.10	8.91	0.181

4.4.11 Effect of agitation on cellulase production

The yeast culture media were shaken at different speed levels to define the role of aeration on cellulase production. The obtained results in Table 10 showed that the agitation stimulated the growth and cellulase production with a maximum at 400 rpm.

4.4.12 Time course of cellulase production

The yeast strain under study was cultivated in media (3.9.14). Different samples were taken during cultivation and analyzed for cellulase activity. The obtained data in Table 11 showed that the activity was found between 6h and 72 h.

Table 10. Effect of agitation on cellulase production.

Agitation (rpm)	pH after cultivation	Biomass dry wt (mg/ml) after cultivation	Cellullase activity (U/ml)
0	7.26	14.81	0.138
100	8.02	50.14	0.145
200	7.84	70.07	0.148
300	8.19	76.55	0.170
400	8.10	86.22	0.177
500	8.00	77.74	0.170

Cellulase was detected after 6 h and remained up to more than 72 h of cultivation. The optimum yield was found at 24 h. The growth corresponded to the cellulase production up and down at all cultivation times.

Table 11. Effect of incubation time on growth and cellulase production.

Incubation time (h)	pH after cultivation	Biomass dry wt (mg/ml) after cultivation	Cellulase activity (U/ml)
6	7.70	32.59	0.016
12	7.80	36.59	0.149
18	7.82	156.66	0.147
24	7.90	163.70	0.170
30	8.30	159.70	0.163
36	8.44	145.40	0.144
48	8.94	81.48	0.143
56	9.90	86.07	0.141
72	9.11	74.14	0.085

4.5 Isoelectric point (pI)

Determination of the isoelectric point (pI) facilitated the subsequent chromatographic purification steps. Isoelectric focusing was also used to test the purity of the enzyme.

The obtained zymogram (Fig. 11) indicated that the pI of the isolated enzyme was between 4.8-5.0

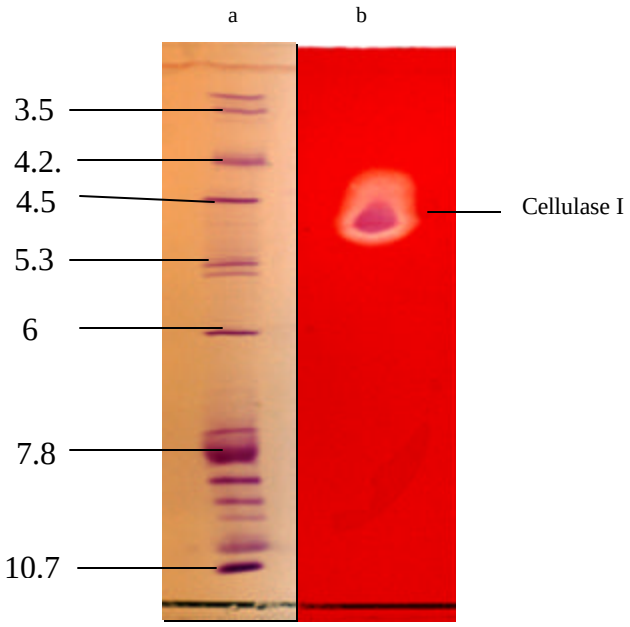

Fig. 11. Isoelectric focusing analysis of the isolated yeast cellulase I.

(a) marker proteins (amyloglucosidase, 3.5 ; glucose oxidase, 4.2 ; trypsin inhibitor, 4.5; ß-lactoglobulin, 5.2-5.3; carbonic anhydrase, 6; myoglobin, 6.9-7.4 ; lectin, 7.8-8.3 ; ribonuclase A, 9.5; and cytochrome C, 10.7), (b) Purified Cellulase

4.6 Apparent molecular mass

Polyacrylamide gel analysis was used to determine the molecular weight of the prepared enzymes. The analysis was carried out under non-denaturing conditions for cellulase determination.

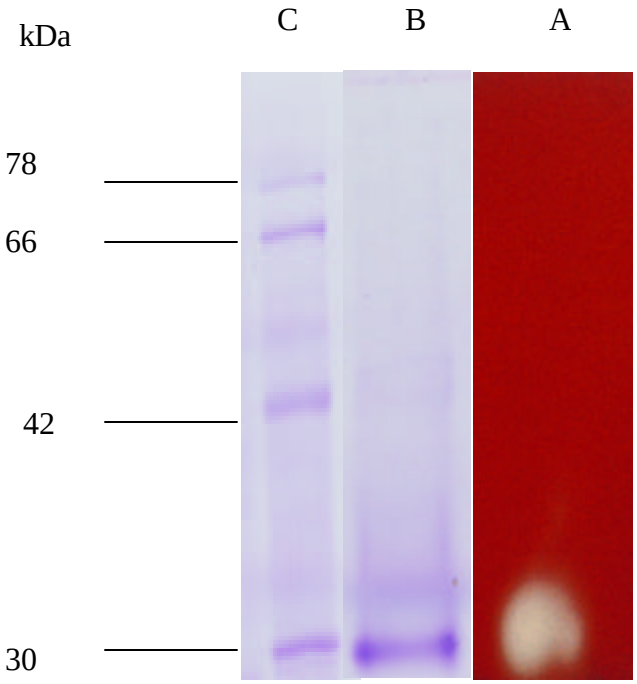

Fig. 12. SDS-PAGE of cellulase I.

Lane A: cellulase I after Congo red staining, Lane B: cellulase I after coomassie blue staining, lane C: molecular mass markers.

The resuls exhibited that the cellulase I has an apparent molecular mass of about 30 kDa Fig.12 while the size of cellulase II was approximately 66 kDa Fig. 13.

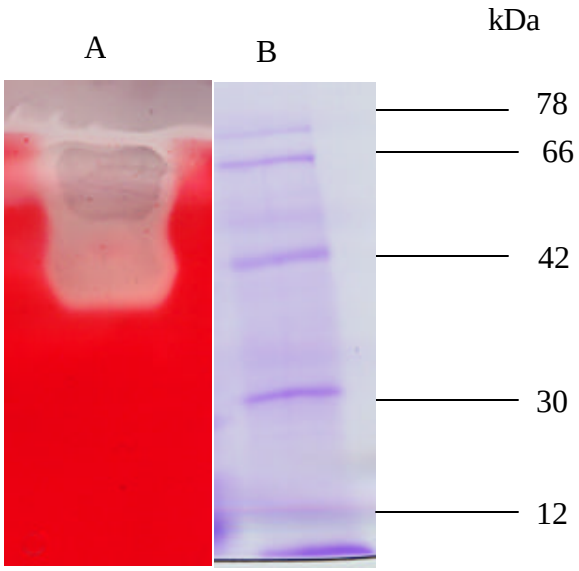

Fig. 13. SDS-PAGE of the cellulase II preparation.

Lane A: cellulase II after Congo red staining, Lane B: molecular mass.

4.7 Purification of cellulase

4.7.1 Crude cellulase preparation

In order to reduce the protein impurities of the culture supernatant, it was brought to 30 % ammonium sulfate saturation and then centrifuged. This step reduced 4896.34 mg /20 l of protein impurities. Also the treatment of supernatant with 80 % ammonium sulfate saturation increased the specific activity to 0.8092 U/mg.

4.7.2 Anion-exchange chromatography

For ion exchange chromatography the working pH should be one unit higher than the pI of the protein. At this pH value the protein will possess a net charge high enough to bind well to the ion exchange resin. However, the cellulase from the yeast strain did not bind to the resin under this condition. Therefore, the separation was tried at different pH values. The best results were achieved at pH 7.6 (Fig. 14).

The concentrated enzyme preparation after dialysis was subjected to a Mono Q HR 5/5 column, which was equilibrated with 20 mM Tris/HCl buffer pH 7.6. The elution was performed with 2 M NaCl in the same buffer. The elution profile (Fig. 14) indicated that the cellulase activity was present in fractions 11-15, which were separated into two peaks (II, III). They eluted at a NaCl concentration of 0.12- 0.2M and 0.2- 0.26 M, respectively and designated as cellulase I and II. The fractions from each peak were pooled, desalted and concentrated. Cellulase I was used for further purification.

4.7.3 Hydrophobic interaction chromatography (HIC)

During HIC nonpolar regions of proteins bind to the resin, separation is carried out by decreasing the salt concentration of the eluent. For further purification the buffer of the concentrated cellulase I sample from the former step (4.7.2) was buffer exchanged by 20 mM sodium phosphate pH 7 containing 1.5 M ammonium sulfate.

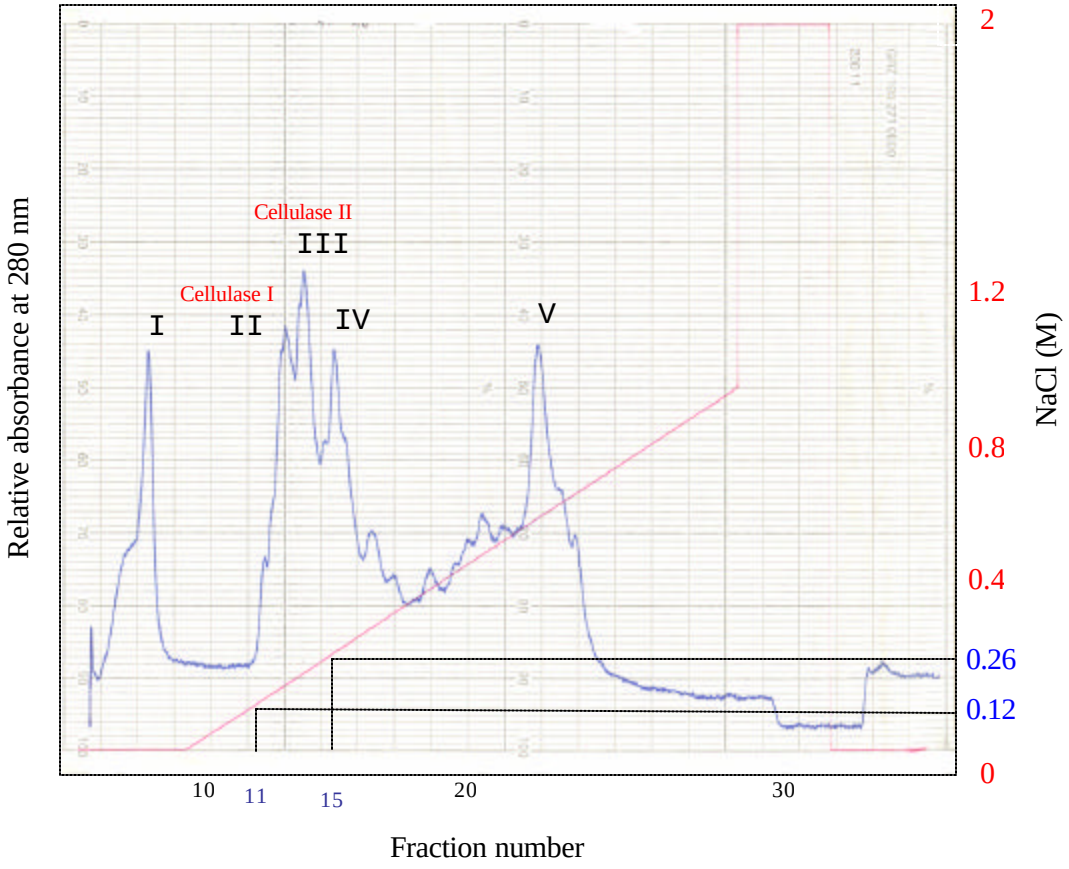

Fig. 14. Elution profile of crude cellulase preparation using Mono Q HR 5/5 anion exchange column.

Column was equilibrated with 20 mM Tris/HCl pH 7.6 and eluted with linear gradient of 2M NaCl in the equilibration buffer at a flow rate of 1 ml/min. Fractions (1 ml) each were collected and assayed for activity. Cellulase I was eluated at 0.12-0.2 M NaCl fractions 11-13. Cellulase II was eluted at 0.2-0.26 M NaCl fractions 14-15.

The sample was applied to Resource ISO column and eluted with above buffer without ammonium sulfate. The elution profile of the cellulase I fraction (Fig. 15) showed that the enzyme was eluted at 0.06 - 1.2 M ammonium sulfate. Activity was detected in fractions 17 – 22. Cellulase I was eluted as a single peak by HIC but SDS-PAGE showed more than one band. Active fractions were pooled and concentrated.

4.7.4 Rechromatography

The concentrated enzyme solution (cellulase I) from HIC chromatography was desalted and the buffer was exchanged by 20 mM Tris/HCl, pH 8. The resulted solution was applied onto a Mono Q column which was equilibrated with 20 mM Tris /HCl buffer pH 8, and eluted with the same buffer containing 2 M NaCl. The elution profile (Fig.16) shows that the enzyme was separated as a single peak, and proved to be a single band on a polyacrylamide gel. Active fractions 22-26 were collected, concentrated, desalted and used as pure enzyme preparation.

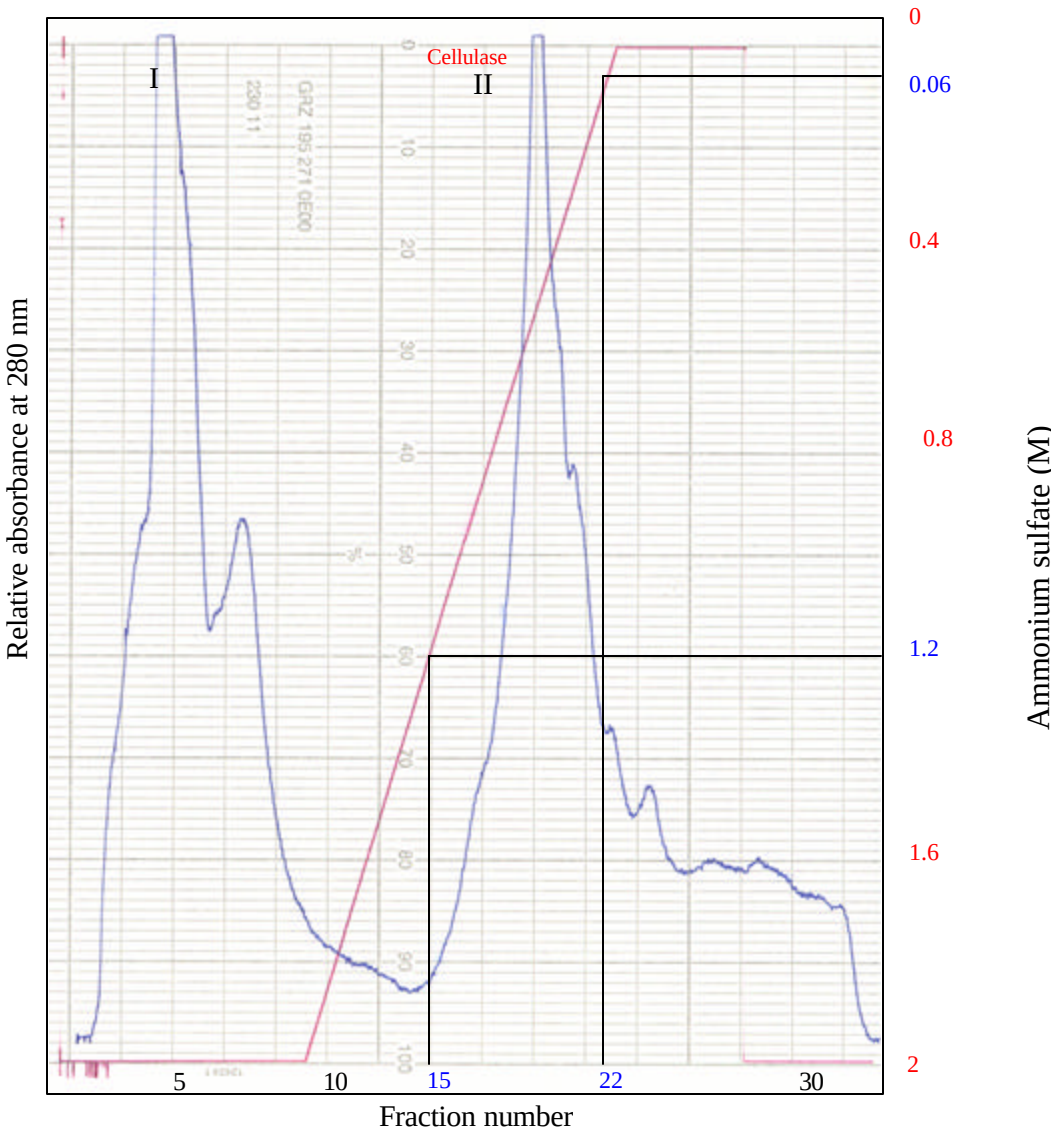

Fig. 15. FPLC elution profile of cellulase I preparation by hydrophobic interaction chromatography. Fractions from Mono Q HR 5/5 column were loaded on Resource ISO column, equilibrated with 20 mM sodium phosphate buffer pH 7 containing 1.5 M ammonium sulfate. The column was eluted with linear gradient of equilibrated buffer without ammonium sulfate at a flow rate 1 ml/min. Fractions 1 ml were collected and assayed for cellulase activity.

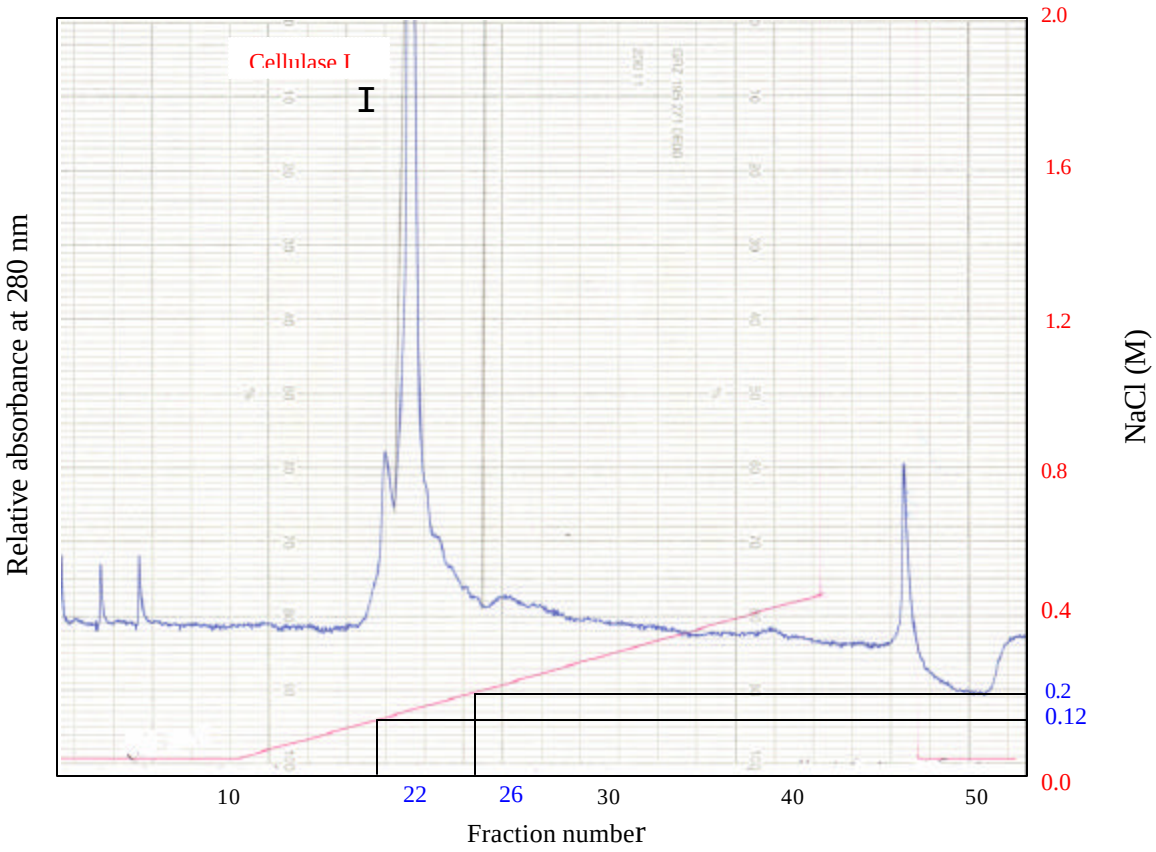

Fig. 16. Rechromatography of cellulase I by using Mono Q HR 5/5 anion exchange column.

Concentrated cellulase I from HIC column was loaded onto the column which was equilibrated with 20 mM Tris/HCl pH 8. Elution was performed with a linear gradient of 2 M NaCl. At a flow rate of 1 ml/min, active fractions were collected and used as pure enzyme preparation.

4.8 Physical and chemical properties of purified cellulase (I)

4.8.1 Effect of pH on enzyme activity

The effect of pH on the enzyme activity was investigated by determination of the activity at various pH values. The used buffer systems were 50 mM: glycine/HCl buffer pH 2-3; sodium citrate buffer pH 4-5; sodium phosphate buffer, pH 6-7; Tris/HCl buffer pH 8 and glycine/HCl buffer pH 9-11. The results were reported in Table 12 showed that the enzyme was active over a broad pH range from pH 3 to 9. The significant activity was detected at a pH interval between 4 and 8. The highest activity was found at pH 5.

Table 12. Effect of pH on cellulase I activity.

pH	Relative activity (%)
2	03.83
3	22.44
4	86.73
5	100.00
6	95.844
7	82.08
8	73.58
9	55.14
10	02.00
11	00.00

4.8.2 Effect of pH on cellulase I stability

The pH stability of cellulase I was investigated by measuring the residual activities after 3 h of incubation at 4 ^{o}C with buffers of different pH values ranging from 2 to 11. The obtained results in Table 13 indicated that the enzyme retained more than 80 % of its normal activity after incubation at pH values from 2 to 9. The highest stability was obtained at pH 5 and activity was completely lost at pH 10.

Table 13. Effect of pH on cellulase I stability.

pH	Relative activity (%)
Control	100
2	81.71
3	97.48
4	98.28
5	99.48
6	94.57
7	90.05
8	84.00
9	80.00
10	00.00

4.8.3 Effect of temperature on cellulase I activity

The temperature profile of the purified enzyme was studied by measuring the activity at different temperatures from 4^{o}C to 70 ^{o}C.

The results given in Table 14 showed that the enzyme was active over a broad range of temperatures (4 ^{o}C - 60 ^{o}C). The activity was higher than 70 % of the maximal activity at the temperatures 20 ^{o}C and 50 ^{o}C. The temperature optimum for the enzyme was at 40 ^{o}C.

Table 14. Effect of temperature on cellulase I activity.

Temperature (^{o}C)	Relative activity (%)
4	47.36
10	54.45
20	75.15
30	79.58
40	100.00
50	77.56
60	36.18
70	00.00

4.8.4 Effect of temperature on enzyme stability

The purified cellulase I solution was incubated in 50 mM sodium citrate buffer at different temperatures and the remaining activity was determined with the DNS method. The results presented in Table 15 showed that the enzyme was highly stable at temperatures up to 30 °C under the described conditions. It retained more than 90 % of its activity after incubation at 50 °C, whereas the enzyme was nearly inactive at temperatures above 60 °C. Furthermore, the stability was tested by incubating the enzyme in 50 mM sodium citrate buffer pH 5 at 4 °C and -20 °C in the presence of NaN_3 (0.2 g/l) to prevent microbial growth. No loss of the enzyme activity was observed during the incubation at 4 °C for 48 h. The enzyme retained full activity after storage at -20 °C for 30 months.

Table 15. Effect of temperature on cellulase stability.

Temperature (°C)	Relative activity (%)
Control 4	100.0 103.8
10	102.1
20	98.5
30	100.2
40	96.8
50	90.5
60	0.79
70	0.18

4.8.5 Effect of various chemicals on enzyme activity.

The enzyme activity was investigated in the presence of different reagents with the DNS method. The results presented in Table 16 indicated that no effect on the activity was found with surfactants i.e. Tween 40, Tween 80, pluronic F68 and silicone antifoam. In contrary, Triton X-100 decreased the activity up to 75 %.

The influence of reducing agents was as follows:

- No effect on the activity was found in the presence of cysteine hydrochloride.
- Weak inhibition was observed in the presence of pyroglutamate, ectoin and hydroxyectoin.
- Strong inhibition of 30 % or 50 % was caused by proline and 2-mercaptoethanol, respectively.

Chelating agents such as EDTA and other reagents such as NaN_3, iodoacetate and glycerol had no influence on the activity, whereas SDS appeared to be a potent inhibitor.

4.8.6 Effect of metal ions on enzyme activity.

The effect of metal ions on the enzyme activity was examined by measuring the activity in the presence of 1 mM of each metal ion. From the data given in Table 17 it could be noticed, that the metal ions had a wide variety of effects on the activity.

Table 16. Effect of various chemicals on cellulase activity.

Chemical	Relative activity (%)
None	100.00
Pyroglutamate	93.31
Proline	70.75
Ectoin	98.78
Hydroxyectoin	94.94
EDTA	99.10
Glycerol	103.30
2-Mercaptoethanol	52.89
Cysteine hydrochloride	102.94
Tween 40	106.18
NaN_3	100.55
Tween 80	104.46
Pluronic F68	102.94
TritonX-100	75.53
Silicone antifoam	105.39
Iodoacetate	102.79
Glutathione	95.58
SDS	1.50

a) Stimulation: $CuCl_2$ and $MnCl_2$.

b) No effect: $FeCl_3$, NaCl, $BaCl_2$, LiCl, $MgCl_2$ and $CaCl_2$.

c) Weak inhibition effect: $CdCl_2$ and $Pb(CH_3COO^-)_2$.

d) Strong inhibition: $AgNO_3$.

e) Complete inhibition: $HgCl_2$.

Table 17. Effect of metal ions on cellulase I activity.

Metal ion	Relative activity (%)
None	100
$CdCl_2$	88.12
$BaCl_2$	103.12
$CaCl_2$	94.40
$FeCl_3$	101.80
KCl	97.151
$MnCl_2$	108.89
$CuCl_2$	125.98
$MgCl_2$	97.05
LiCl	109.28
$Ag\ NO_3$	32.37
NaCl	101,01
$Pb(CH_3COO^-)_2$	94.77
$HgCl_2$	00.00

4.8.7 Effect of organic solvents on enzyme activity

The effect of organic solvents on the enzyme activity were investigated by using reaction mixtures containing 20 % (v/v) of each tested solvent. Data in Table 18 showed that all tested organic solvents had no stimulation effect on the activity. Instead, the tested organic solvents had inhibitory effects except toluene which had no influence on the activity. The suppression of enzyme activity by organic solvents may be categorized into four classes:

(a) No inhibition: e.g. toluene

(b) Significant inhibition (up to 50%): e.g. methanol, ethylenglycol and dimethylsulfoxide.

(c) Strong inhibition (more than 75 %): e.g. acetone.

(d)Total inactivation: e.g. ethanol and acetonitrile.

Table 18. Effect of organic solvents on cellulase I activity.

Solvent	Relative activity (%)
None	100.00
Methanol	49.29
Ethanol	00.00
Ethylenglycol	55.64
Toluene	100.5
Acetonitrile	0.00
Dimethylsulfoxide	51.72
Acetone	23.27

4.8.8 Inhibitory effect of oligosaccharides

The inhibitory effect of a broad range of oligosaccharides on the enzyme activity was examined in the presence of different concentrations of each saccharide.

From the results in Table 19 it can be concluded that the inhibition effect of oligosaccharides was as follows:

- •No inhibition: raffinose, sucrose and melizitose.
- •Inhibition with different degree: lactose, cellobiose, maltose and gentiobiose.

Total inhibition: lactose, 2 mM; both cellobiose and maltose, 2.6 mM. While gentiobiose was the weakest inhibitor which showed total inhibition at 2.8 mM.

Table 19. Effects of oligosaccharides on cellulase I activity.

Oligosaccharides (mM final concentration)	Relative activity (%)						
	Cellobiose	Lactose	Sucrose	Gentiobiose	Raffinose	Melizitose	Maltose
0.0	100	100	100	100	100	100	100.0
0.6	10.01	90.06	100	102.5	100	100	102.0
0.8	99.98	85.91	100	95.01	100	100	103.1
1.0	84.24	61.74	100	87.06	100	100	93.50
1.4	78.51	44.33	100	75.59	100	100	78.70
1.6	71.00	33.14	100	68.94	100	100	61.54
1.8	47.89	21.40	100	58.30	100	100	50.50
2.0	45.98	00.00	100	39.60	100	100	48.38
2.2	23.38	00.00	100	38.02	100	100	30.19
2.4	007.23	00.00	100	36.07	100	100	20.00
2.6	00.00	00.00	100	21.10	100	100	00.00
2.8	00.00	00.00	100	00.00	100	100	00.00

4.8.9 Substrate specificity

The enzyme activity was examined towards several soluble substrates, i.e. CMC, oligosaccharides having ß-linkages with different types and positions and p-nitrophenyl- ß-derivates, and also against insoluble cellulosic substrates.

The specific activities towards the tested substrates were presented in Table 20. The results revealed that the purified enzyme could not hydrolyze any type of glycosidic linkages such as ß- (1-4), ß- (1-3) and ß- (1-6) which existed in the tested oligosaccharides cellobiose, melizitose and gentiobiose, respectively.

The enzyme also was incapable to hydrolyze the tested p-nitrophenyl-ß-derivatives. On the other side, the enzyme was found to be able to hydrolyze all tested cellulosic substrates. The specific activity against the soluble substrate (CMC) was much higher than against insoluble cellulose. Among the insoluble substrates, the enzyme showed highest activity against H_3PO_4-swollen avicel. The enzyme showed also significant activity towards xylan compared with sigmacell and cellulose powder which were poorly cleaved.

Table 20. Substrate specificity of cellulase I

Substrate	Specific activity [U/mg]
CMC	10.078
∗Microcrystaline cellulose	00.017
∗Xylan	0.056
∗Celluose powder	0.006
∗Avicel (H_3PO_4 swollen)	0.145
p-Nitrophenyl-ß-D-glucuronide	00.000
p-Nitrophenyl-ß-xyluronide	00.00
2-Nitrophenyl-ß-D-galactopyranoside	00.000
p-Nitrophenyl-ß-D-glucopyranoside	00.000
p-Nitrophenyl-ß-xyloside	00.000

∗These substrate were treated by ultrasonication for 8 min at 19 Hz

4.8.10 Reaction kinetics

The reaction kinetics of the purified cellulase I were calculated by using enzyme program **(http://www.uni-mainz.de/FB/Biologie/Mikrobiologie/download/download.htm)**.

The enzyme had apparent K_m value of 0.91 % and V_{max} value of 191.98 µmol/min/mg for hydrolysis of CMC.

4.8.11 Saccharification products

To obtain available glucose from cellulosic materials, the enzyme was incubated with acid swollen avicel and CMC in the presence and absence of ß- glucosidase from the same yeast. The reaction products were analyzed by HPLC.

Results in Fig. 17 and 18 showed that the purified cellulase could degrade both soluble (CMC) and insoluble (avicel) cellulosic materials to small chains of ß-(1,4) oligosaccharides. Among the degradation products of avicel it could be observed that the smallest produced oligosaccharide was cellobiose (2 glucose units) and the longest one was cellopentaose (5 glucose units).

It was noticed that no glucose was found among the degradation products of both substrates (CMC, avicel). Glucose was only observed as main product beside cellobiose when purified cellulase I and ß- glucosidase from the same yeast were added Fig. 19. This means that ß - glucosidase from the same yeast acts synergistically with the cellulase to complete the hydrolysis of cellulosic materials to available glucose.

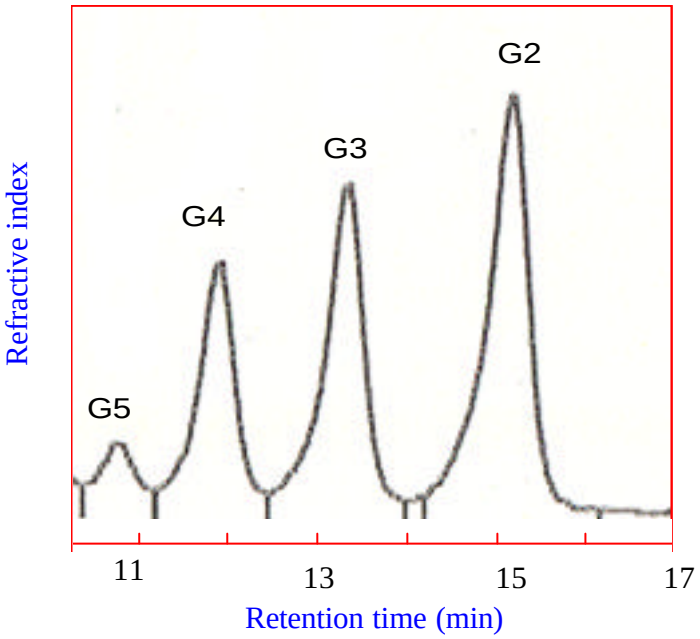

Fig. 17. HPLC chromatogram of degradation products of avicel by cellulaseI

Retention time of each saccharide is indicated on its peak. G2: cellobiose; G3: cellotriose; G4: cellotetraose; G5: cellopentaose. The hydrolysis products of CMC included cellobiose, cellotriose and cellotetraose

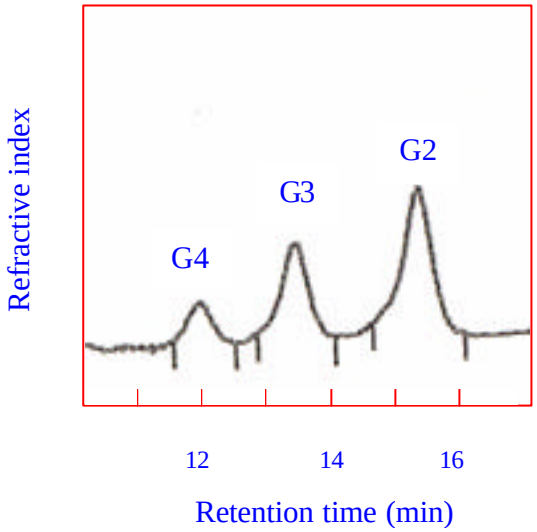

Fig. 18. HPLC analysis of degradation products of CMC after incubation with purified cellulase I.

G2: cellobiose; G3: cellotriose; G4: cellotetraose; G5: cellopentaose.

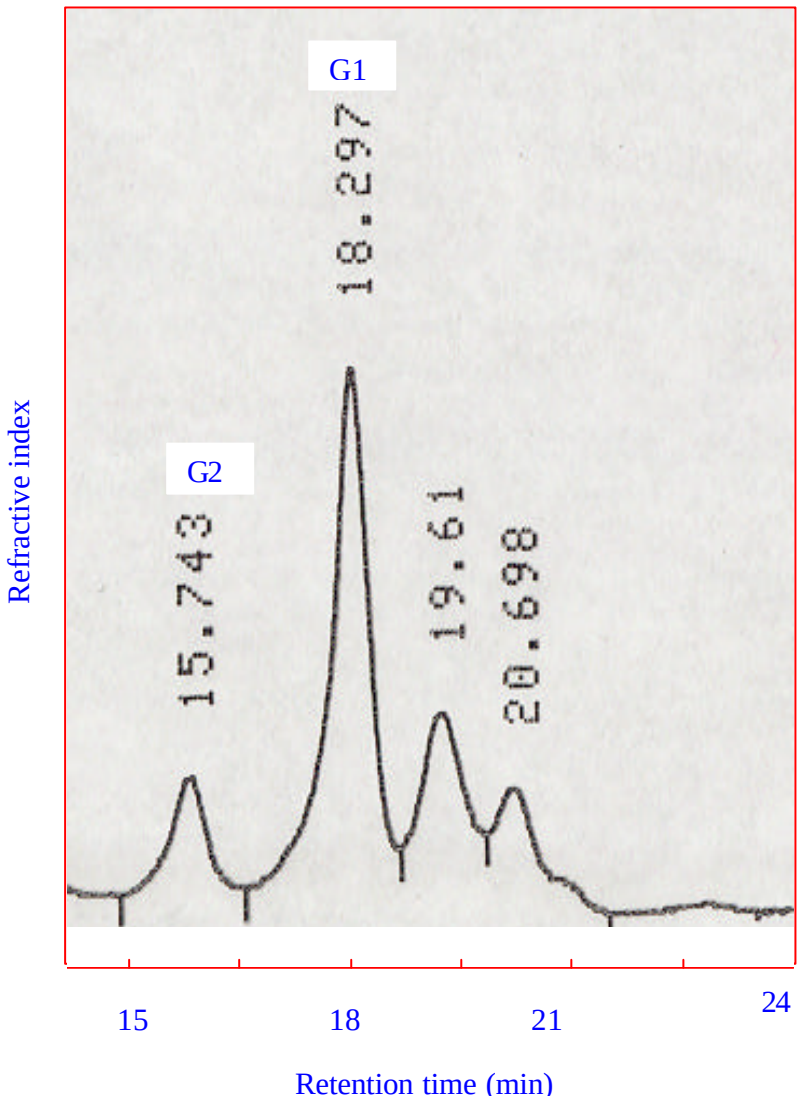

Fig. 19. High-performance liquid chromatography analysis of saccharides produced from avicel by synergistic interaction between cellulase I and ß-glucosidase.

G1: glucose; G2: cellobiose.

5 DISCUSSION

5.1 Optimal conditions for cellulase production

Optimization of the medium for cellulase production by selecting the best nutritional and environmental conditions is important to increase the produced cellulase yield (Gomes et al., 2000)

5.1.1 Optimal temperature

The upper temperatures limit for growth of psychrophilic, mesophilic, thermotolerant and thermophilic yeasts were found to be 20, 35, 42 and 45 ^{o}C, respectively (Arthur and Watson, 1976).

The isolated yeast strain PAG1 in this study was able to survive over a broad range of temperature Fig. 20. The most significant growth and cellulase production were observed between 20 ^{o}C and 35 ^{o}C. The optimal growth of the isolated yeast was determined at 30 ^{o}C. Therefore, the isolated strain was classified as a mesophilic yeast. It was reported that the best temperature for cellulase production is 30 ^{o}C for *Penicillium citrinum* (Olutiola, 1976) and 30 ^{o}C – 37 ^{o}C for *Bacillus* KSM-635 (Ito, 1997). In the case of *Sporotrichum thermophile* maximum production of cellulases occurred at 45 ^{o}C (Coutts and Smith, 1976). On the other hand, *Aspergillus fumigatus* IMI 143864 showed maximum growth and cellulase production at temperature between 30 ^{o}C and 45 ^{o}C (Stewart and Parry, 1981). However, the optimum cultivation temperature for endo-ß-glucanase production by *Rhodotorula glutinis* is 20 ^{o}C (Oikawa et al, 1998).

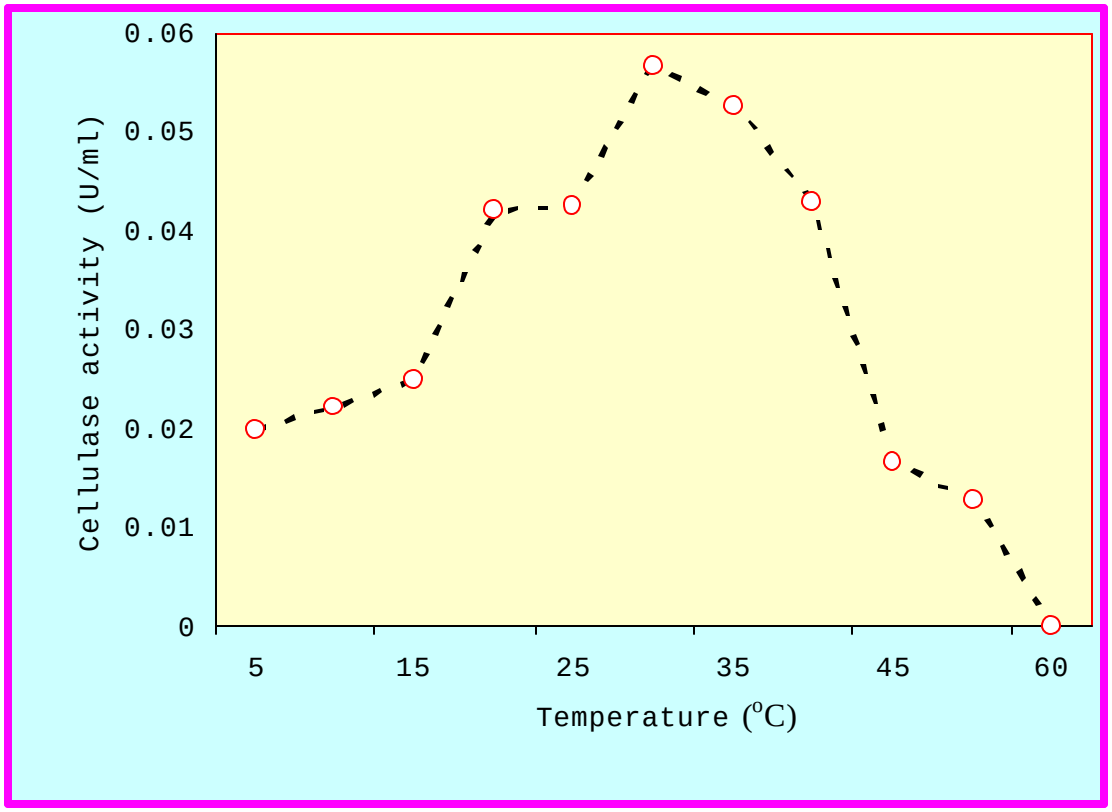

Fig. 20. Effect of incubation temperature on cellulase production.

5.1.2 Optimal carbon source

The obtained results indicated that cellulase production was stimulated in the cultivation medium by soluble and insoluble cellulose substrates. Cellulase induction depended on the presence of low levels of cellulase (constitutive enzyme) in the uninduced organism. This basal cellulase

activity would digest cellulose releasing oligosaccharides that could enter the cell and trigger expression of cellulases (Carle-Urioste et al., 1997).

The obtained results showed that CMC stimulated higher cellulase yield compared to avicel, and the poorest cellulase production was detected with amorphous cellulose as a carbon source. This may be explained on the basis of absorption of the enzymes onto cellulose (Stewart and Parry, 1981). Similarly, CMC also stimulated the highest yield of cellulase in the case of *Penicillium citrinum*, when it was the sole carbon source (Olutiola, 1976). CMC was also required for the cellulase production by members of genus *Bacillus* (Ito, 1997). Lactose and CMC were optimal inducers of cellulase production by *Myceliophthora thermophila* D-14 (Sen et al, 1983). In contrary, amorphous celluloses stimulated higher yields of cellulase from *Aspergillus fumigatus* (Stewart and Parry, 1981). Microcrystalline cellulose induced the highest yield of cellulase when it was used in grown cultures of *Thermomonospora fusca* (Spiridonov and Wilson, 1998). The highest cellulases produced by *Schizophyllum commune* were recorded with thiocellobiose, but CMC, cellobiose and avicel as inducers stimulated lower enzyme yields (Rho et al., 1982).

In the order, D-glucose followed by D-saccharose, glycerol and finally D-fructose, was the best carbon source for cellulase production by *Rhodotorula glutinis* (Oikawa et al, 1998).

5.1.3 Carbon source concentration

The results demonstrated that medium viscosity CMC stimulated higher yield than high viscosity CMC. The yield was decreased in the presence of high concentration of MV-CMC and the growth was approximately stable. The high viscosity medium led to the cease of the growth. This is probably due to the high viscosity of the medium, which decreases the oxygen supply to the cells. Oxygen is necessary for synthesis of cell membrane components (sterols, nonsaturated fatty acids) in the yeast. High viscosity leads to retard cell division, resulted in low production metabolism and cellulase excretion (Fritsche, 1999).

5.1.4 Optimal nitrogen source

The obtained data (Fig. 21) revealed that the organic nitrogen compounds stimulated higher growth and cellulase production than inorganic compounds. This finding probably could be attributed to the lack of amino acids in inorganic compounds (Rakshit and Sahai, 1989). On the other hand, the maximum cellulase yield was found with peptone, which may function as a source for certain essential amino acids to enhance enzyme production (Rakshit and Sahai, 1989). Similar results were reported with the wild strain of *Chaetomium globosum* which produced maximum yield of cellulases in the presence of peptone as nitrogen source followed by yeast extract, urea, KNO_3 and $(NH_4)_2SO_4$ (Umikalsom et al, 1997). In agreement with the present results, soymeal was found to be the best organic nitrogen

source for cellulase production by *Thermoascus aurantiacus* due to the presence of essential amino acid, vitamins and minerals (Gomes et al, 2000). Results also showed that meat extract stimulated higher growth compared to peptone but lower cellulase yield. This may be due to the release of more proteolytic enzymes in the culture medium by meat extract, which attacks the cellulase. D'Souza and Volfova (1982) indicated that proteolytic enzymes might decrease the cellulase level in culture media of *Aspergillus terreus.* These reported data supported our results obtained in this study.

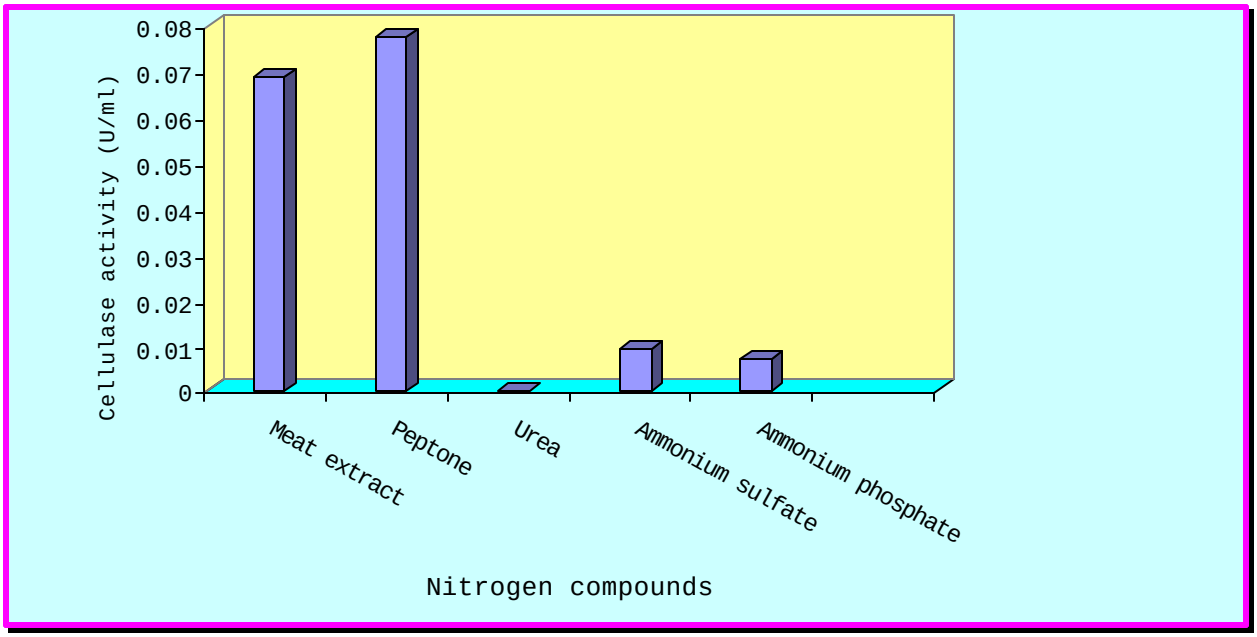

Fig. 21. Effect of nitrogen compounds on cellulase production.

5.1.5 Concentration of nitrogen source

Higher concentrations of peptone in the culture media were followed by an increase of cellulase production. The optimal peptone concentration was shown to be 0.5 and 0.8 % (w/v). These results were in agreement with those of Umikalsom et al. (1997), Rakshit, and Sahai (1989). They found that 0.6 % (w/v) peptone induced the highest cellulase production in the case of *Chaetomium globosum* and *Trichoderma reesei*. The published data also revealed that above the optimal concentration of peptone, cellulase yield decreased. This phenomenon was also observed from Umikalsom et al. (1997). They reported that a yeast extract and peptone concentration above 9 g/l were inhibitory to cellulase production by *Chaetomium globosum.*

One assumes that an excess of peptone in the culture media may induce proteases that hydrolyzes the cellulase protein.

5.1.6 Optimal pH value of culture

Yeast strain was able to grow at a wide initial pH between 3 and 10. After cultivation, the pH was about 8.5 in all media. This proved that the organism was able to optimize the pH in the culture medium for its growth, but no explanation was concluded. Results (Fig. 22) also revealed that the optimal growth and cellulase production was at pH 7. On comparison with other organisms, the optimum initial pH value 7 of the yeast under study was found to be the same as of various cellulolytic organisms such as *Aspergillus fumigatus*, *Neurospora crassa* and *Sporotrichum thermophile* (Stewart and Parry, 1981; Eberhart et al., 1977; Coutts and Smith, 1976). The optimal pH

value of other cellulolytic organisms varied from acidic condition such as *Trichoderma reesi* strain QM-9414 (pH 3.5; Krishna et al., 2000), *Trichoderma reesi* strain MQ 6a (pH 2.8; Sternberg and Mandels, 1979) and *Rhodotorula glutinis* and *Aspergillus terreus* (pH 5; Oikawa et al., 1998; D'Souza and Volfova, 1982) to alkaline conditions such as *Bacillus* sp strain KSM-s237 (9 - 12 ; Hakamada et al, 1997).

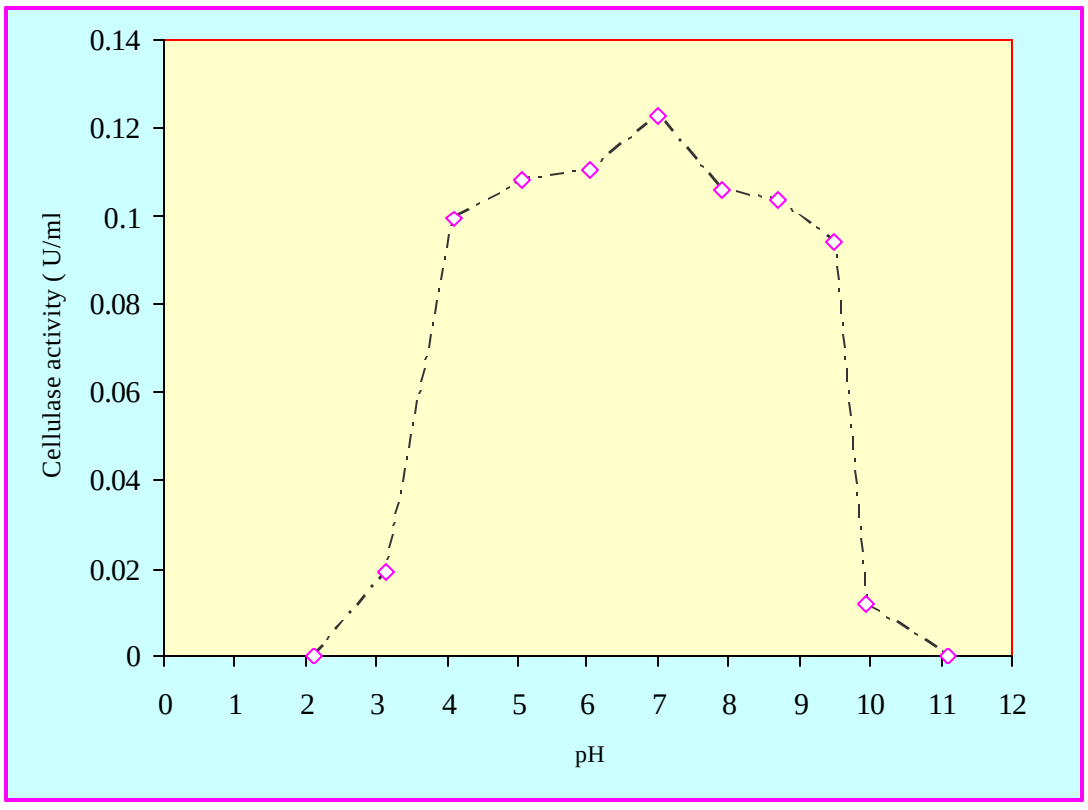

Fig. 22. Effect of growth medium-pH on Cellulase production.

5.1.7 Surfactants effect

All tested surfactants (3.9.9) enhanced cellulase production. Highest yield was induced by Tween 80 (0.5 % v/v). At higher concentration of Tween 80 (>0.5 %; v/v) the cellulase yield did not increase. The stimulatory effect of surfactants may be a consequence of its action on cell membranes causing increased permeability and /or by promoting the release of cell-bound enzymes. On the other hand, the lower stimulatory effect was found with pluronic F68 and silicone antifoam (Fig. 23). This may be due to a decrease in oxygen supply, resulting a diminution of growth (Pardo, 1996). In accordance with the present results, Tween 80 at a concentration of 0.22 (v/v) was the optimal concentration for the production of cellulase by *Nectria catalinensis* (Pardo, 1996). The cellobiase was optimally produced by *Aspergillus niger* A 20 in the presence of 0.2 % (v/v) Tween 80 (Abdel-Fatth et al., 1997). In order to induce a high cellulase production 0.1% and 0.2 % (v/v) of Tween 80 were added to the cellulase production media of *Trichoderma reesei* strain QM-9414 and *Streptomyces flavogriseus* (Krishna et al., 2000; Ishaque and Kluepfel, 1980), respectively.

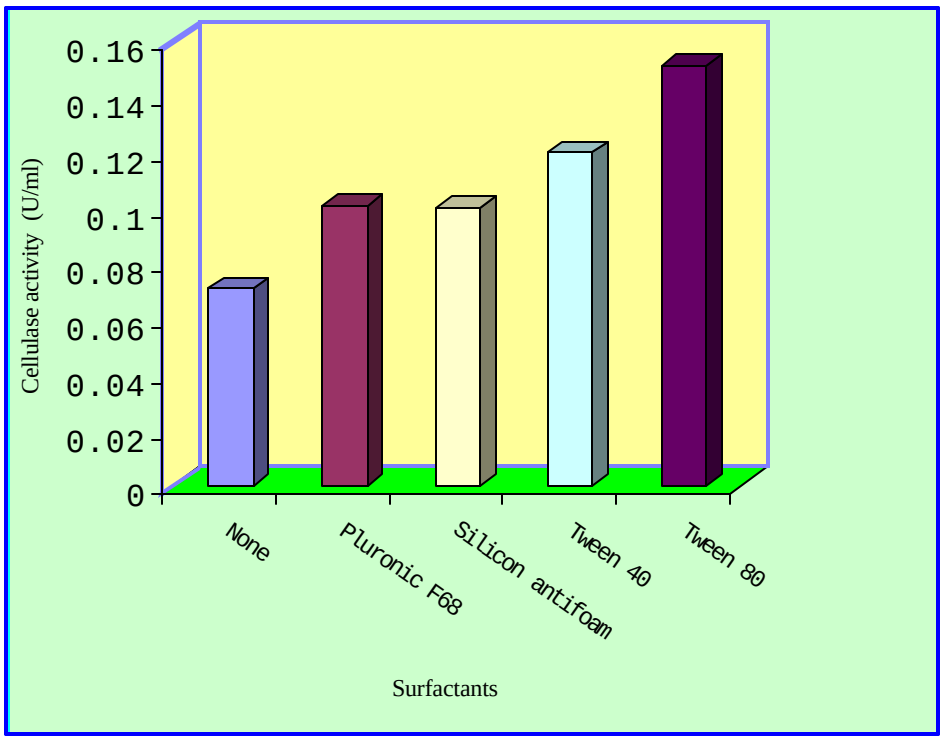

Fig. 23. Effect of surfactants on growth and cellulase production.

5.1.8 Induction of cellulase by lactose

The results revealed that the synthesis of cellulase by the yeast under study is inducible by oligosaccharides being in agreement with the results of Rho et al., (1982), Sen et al., (1983) Wood et al., (1984), Morikawa et al., (1995), Carle-Urioste et al., (1997).

The induction effect of the tested saccharides (Fig. 24) did not depend on the linkages e.g maltose possessing an a -(1,4) linkage exhibited the same effect as lactose and cellobiose, which have ß- (1,4) linkages.

On the other hand, sucrose and raffinose possessing a- and ß-linkage did not induce any cellulase production.

It is also evident from the results that the chain length of the tested saccharides did not play a role in the enzyme induction process.

Therefore, it would not be unjustifiable to assume that an inducer should have a- or ß-linkages or special molecule structure. However, this may not be considered as only criterion, but may be one of the major factors responsible for cellulase induction (Sen et al., 1983).

It is noteworthy to pinpoint that the examined saccharides had a reducing or non-reducing end. All reducing sugars provoked the secretion of the cellulase and all nonreducing sugars did not stimulate cellulase production. From these results, it is suggested that the reducing end of the oligosaccharides is required for the enzyme induction process.

The maximum cellulase production was induced in the presence of lactose followed by cellobiose. These results are in agreement with that of Sen et al., (1983). They found that lactose and cellobiose were the best inducers for cellulase production by *Myceliophthora thermophila* strain D-14. Similar results were reported by Geimba et al., (1999). They indicated that cellulase production by *Bipolaris sorokiniana* was stimulated by lactose. Also lactose, starch and cellobiose induced the cellulolytic and xylanolytic enzymes production by *Piromyces* sp. (Teunissen et al., 1992). Thiocellobiose and cellobiose induced the cellulase production by *Schizophyllum commune* (Rho et al., 1982). The highest activity against filter paper produced by *Aspergillus fumigatus* was induced by filter paper and lactose (Ximenes et

al., 1996). Cellobiose was the best inducer for cellulase production by *Neurospora crassa* (Eberhart et al., 1977).

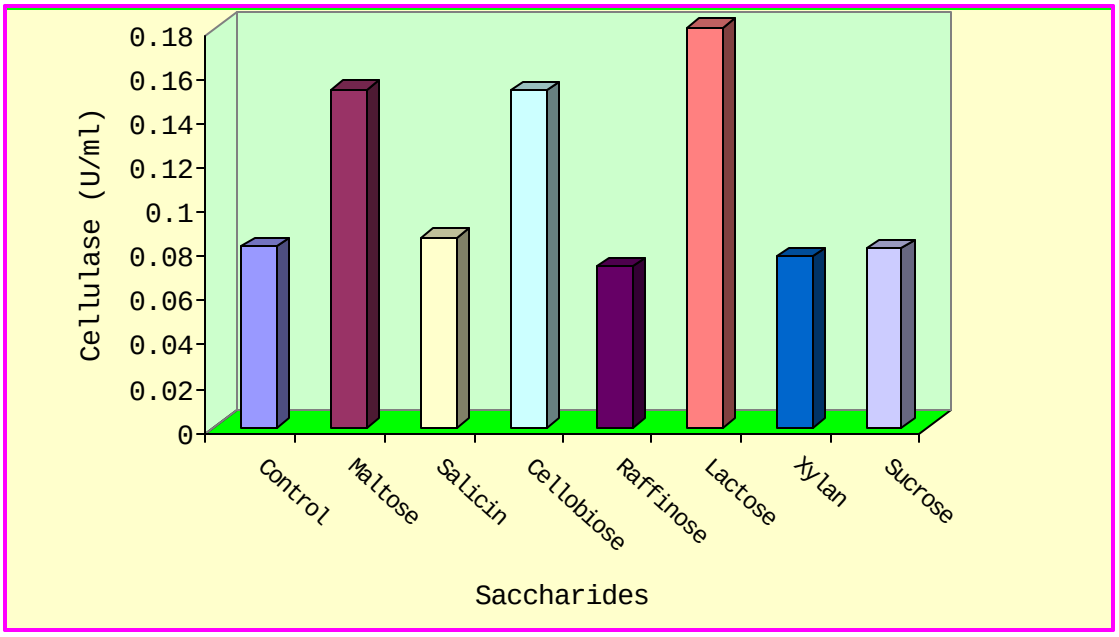

Fig. 24. Induction of cellulase in growth medium by different saccharides.

5.1.9 Inducer concentration

The induction of cellulase was directly proportional of concentration of lactose up to 1 % (w/v). Eriksson and Hamp (1978) reported that when degradable inducer such cellobiose is used, the inducing effect in *Sporotrichum pulverulentum* will disapper because of the depletion of the

inducer. Increasing the inducer concentration will result in catabolite repression due to the accumulation of glucose (Rho et al., 1982). Accordingly, it could be suggested that the increase of lactose higher than 1 % may lead to similar results in the induction of cellulase in the case ofthe studied yeast.

5.1.10 Culture agitation

The obtained results in Fig. 25. revealed that both growth and cellulase production are highly depended on the agitation rate. This may be explained by the fact that the agitation increased the dissolved oxygen in the medium, which is necessary for cell membrane components (sterol, non-saturated fatty acid) and uniform distribution of the medium contents such as foodstuffs and catabolites (Fritsche, 1999). This prevents the repression through the catabolite. This observation is in accordance with the results of Wood et al., (1984). They reported that the extracellular catabolite accumulation might be a factor in endoglucanase repression in case of *Thermomonospora curvata*. Mountfort and Asher (1985) reported that CMCase was improved by shaking the culture media of the anaerobe *Neocallimastix frontalis* PN-1. Higher agitation rates favored the production of xylosidase, arabinofuranosidase and glucosidase by *Thermomyces lanuginosus* strain SSBP, whereas the lower agitation rates favored xylanase production (Singh et al., 2000).

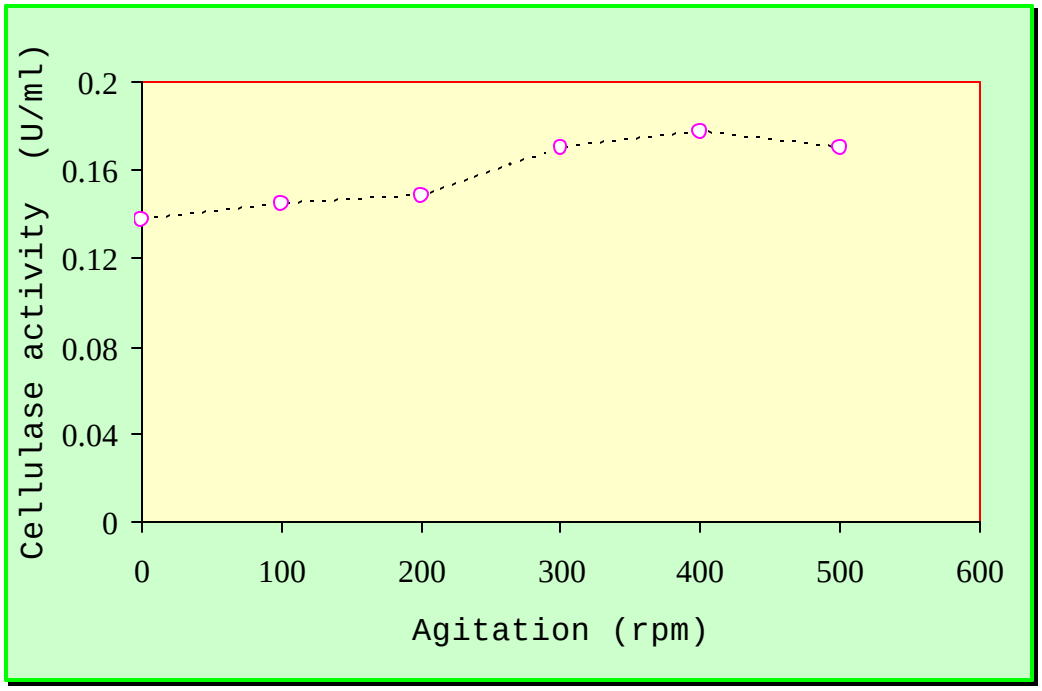

Fig. 25. Effect of agitation on growth and cellulase production.

5.1.11 Cultivation time

Production of cellulase was detected after a cultivation time of 6 h and reached its maximum level after 24 h of cultivation and then starts to decline (Fig. 26). This is probably mainly due to the stop of the growth and release of proteases into the medium during the later growth phase of the yeast. This time course of production of cellulase is shorter compared to other organisms. *Streptomyces flavogriseus* produced maximum cellulase yield after 72 h of incubation at 30 ^{o}C (Ishaque and Kluepfel, 1980).

The cellulase yield of *Sporotrichum thermophile* reached its maximum at 45 ^{o}C between 2 and 4 days (Coutts and Smith, 1976).

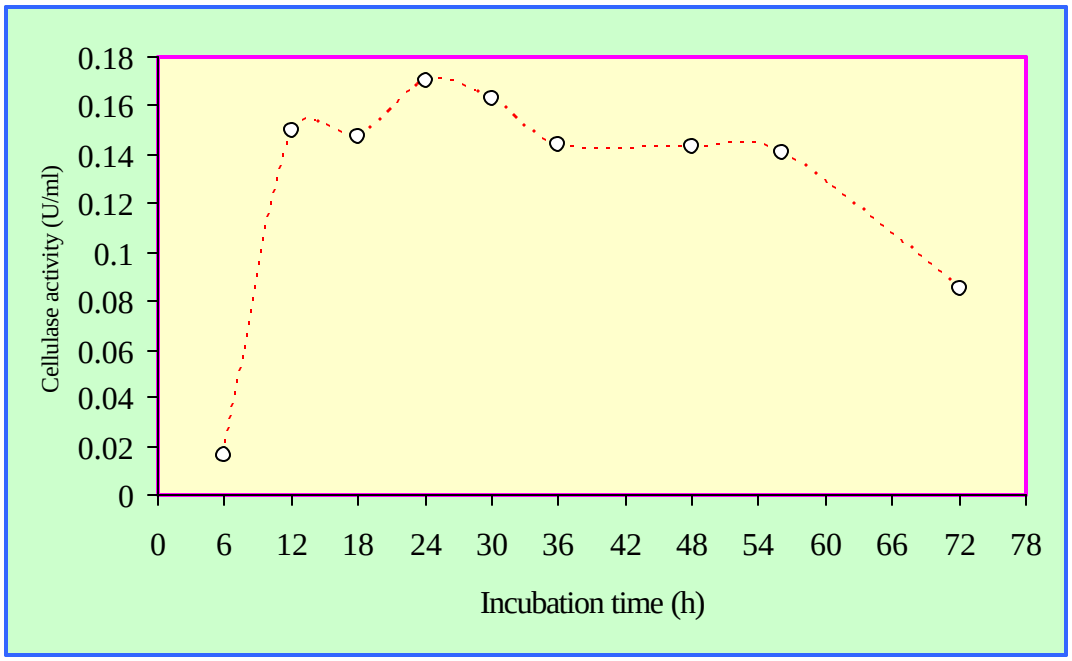

Fig. 26. Effect of incubation time on growth and cellulase production.

5.2 Isoelectric point (pI)

The protein separation by IEF is achieved by loading the protein in a pH gradient generated by an electric field. Under these conditions, the protein migrates until it reaches a position in the pH gradient at which the positive charges of a protein equal the negative charges at the isoelectric point, (pI) (Bollag et al., 1996).

The isoelectrophoretic (IEF) analysis of the cellulase protein in this study showed a pI between 4.8 and 5.0. This means that a positive net charge of the protein. Therefore, the presence of the amino acids arginine, histidine and lysine on the protein surface should be high (Bollag et al., 1996). Since the pI of the cellulase protein is acidic (4.8-5.0), it can be expected that the enzyme is reasonably stable under alkaline conditions (Oikawa et al., 1998). Compared to the pI of previously reported cellulases, these are acidic cellulases such as that from *Bacilluls* sp No1139 (3.1), *Bacillus* sp KSM 237 (3.8), *Clostridium stercorarium* (3.9), *Streptomyces flavogriseus* (4.15), *Trichoderma reesei* (4.75) and *Bacillus circulans* (5.4) (Fukumori et al., 1985, Hakamada et al., 1997, Bronnenmeier et al., 1991, Mackenzie et al., 1984, Ogawa et al., 1991, Kim, 1995), while the pI of the cellulases from *Rhodotorula glutinis* KUJ2731(8.57) was alkalophilic (Oikawa et al., 1998).

5.3 Summary of the purification steps

The purification steps of the cellulase are summarized in Table 21 The specific activity of the enzyme in the culture supernatant was very low (0.0722 U/mg). Treatment of supernatant with 30 % ammonium sulfate saturation precipitated 34 % of the total protein of the culture supernatant. The precipitate protein was considering impurities protein.

After ammonium sulfate precipitation (80 % saturation), the specific activity was increased by 11.2 fold. Two cellulase peaks were obtained after

Table. 21. Summary of the purification steps of cellulases produced by isolated yeast.

Step	Total protein (mg/20l)	Total activity (U/20l)	Specific activity (u/mg)	Purification (Fold)	Yield (100 %)
Culture supernatant	14400	1040	0.0722	1	100
30 % saturation ammonium sulfate	4896.34	51.09	0.0104	-	-
80 % saturation ammonium sulfate	976.70	790.4	0.8092	11.28	76.0
Anion exchange chromatography Mono Q HR 5/5 Cellulase I Cellulase II	 132.88 144.48	 188.5 240.1	 1.418 1.661	 19.63 23.00	 - -
Hydrophobic interaction chromatography Resource ISO Cellulase I	 10.504	 107.261	 10.211	 138.50	 -
Rechromatography Mono Q HR 5/5 Cellulase I	 2.451	 88.481	 36.1	 500	 -

ion exchange chromatography, which were further purified by hydrophobic interaction chromatography.

Cellulase I and cellulase II were enriched 19.63 and 23.00 fold respectively. Only cellulase I was obtained as pure enzyme after an addition purification step. Generally, the loss of cellulases yield after each purification step was markedly high.

The highest loss of the yield was observed in the first three purification steps, after that the yield was gradually increased to reach 80 % at the end of the purification stages.

5.4 Characteristics of cellulase

5.4.1 pH optimum

The purified cellulase I from the studied yeast can be considered as unsusceptible to acidic and alkaline conditions because it showed activity in a broad range of pH between 3 and 9. The increase or decrease of pH values over or below the optimal value of 5.0 was not followed by a rapid lost of activity, because it kept more than 86 % of its activity at a pH value of 4. It possessed 1/5 of its activity at pH 3 (Fig. 27). The enzyme also showed high capability at the alkaline side, whereas it kept 55 % of its activity at pH 9. The results are in agreement with those obtained from *Chalara paradoxa* and *Bipolaris sorokiniana*, which showed an optimal activity at pH 5 (Lucas et al., 2001; Geimba et al., 1999).

On the other hand, *Trichoderma reesei*, *Thermoascus aurantiacus* and *Bacillus circulans* were more on the acid side (pH 4.5) (Krishna et al., 2000; Gomes et al., 2000; Kim, 1995) The endo-ß-glucanase from the psychrotrophic yeast *Rhodotorula glutinis* strain KuJ 2731 was active between pH 2 - 7 with optimal activity at pH 4.5 (Oikawa et al., 1998).

On the other side, more alkaline cellulases than the studied enzyme were reported such as the cellulases from *Neocallimastix frontalis* and *Bacillus* sp. showed an optimal pH value at 6.0 (Mountfort and Asher, 1985; Kricke et al., 1994). Also maximal activity of avicelase II from *Clostridium stercorarium* is observed between pH 5 and 6 (Bronnenmeier et al., 1991). While cellulase from the alkalophilic *Bacillus* sp. No. 1139 was most active at pH 9 and still retained some activity at pH 10.5, it showed no activity at pH 6 (Fukumori et al., 1985). Also a thermostable alkaline cellulase from *Bacillus* sp KSM-S237 had a pH optimum of 8.6 - 9.0 (Hakamada et al., 1997).

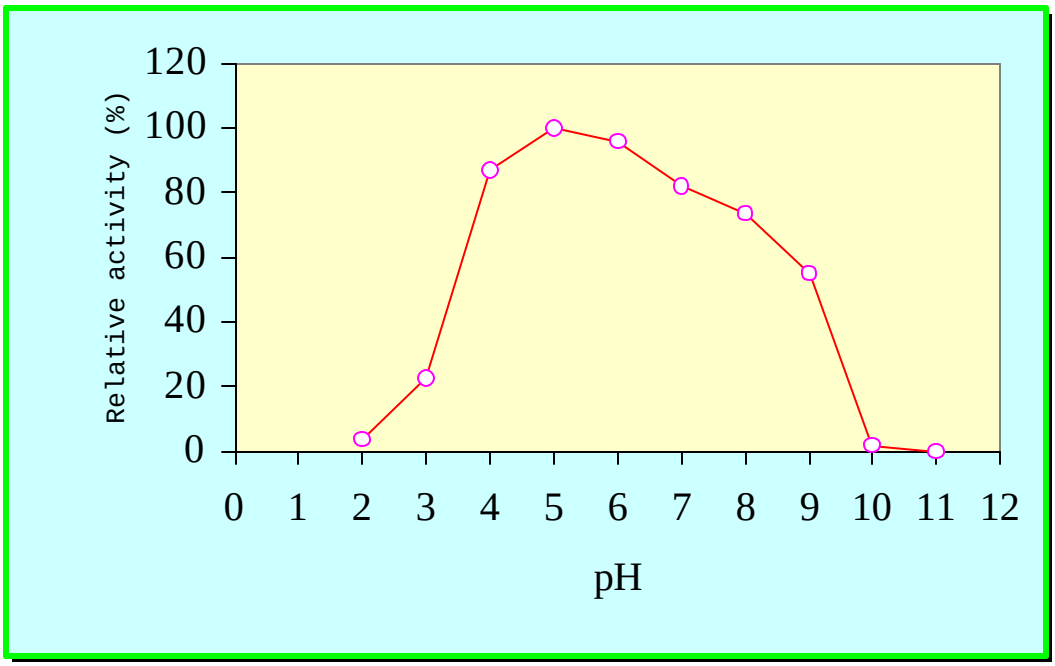

Fig. 27. Effects of pH on cellulase I activity.

5.4.2 pH stability

The purified cellulase I was stable at acidic and alkaline conditions (Fig. 28). It retained more than 80 % of its original activity at pH 2 and 9. It could be observed that the stability range under the acidic condition is larger than that under the alkaline condition. In comparison with other cellulases, which were previously described, the endo- ß-glucanase from the yeast *Rhodotorula glutinis* was fairly stable at a pH range between 2 and 9. It was highly stable in acidic conditions and even after incubation with 50 mM glycin/HCl buffer at pH 2 and 30 ^{o}C for 60 min, more than 75 % of its maximum activity was retained (Oikawa et al., 1998). On the other hand, the CMCase from the alkalophilic *Bacillus* sp No 1139 was stable over a pH range

between 6 and 11. When the enzyme was incubated for 24 h at 4 ^{o}C in 0.2 M sodium phosphate buffer (pH 6 - 8); glycine/NaCl/NaOH buffer (pH 8.5 - 10.5) and KCl/NaOH buffer (pH 11-12.8) (Fukumori et al., 1985). However, an endoglucanase from *Thermoascus aurantiacus* showed stability at pH (4 - 8) for 48 h at 50 ^{o}C (Gomes et al., 2000). Endoglucanase from *Chalara paradoxa* retained > 90 % of their activity after incubation at pH 8 or 9 for 30 min (Lucas et al., 2001). The residual activity of alkalic cellulase from *Bacillus stearothermophilus* remained 90 % of its initial activity after treatment at pH 12, while the remaining activity of the neutral cellulase was about 80 % (Kume and Fujio, 1991). The cellulase from *Bacillus* sp. KSM-635 was stable at pH 6-11 at 5 ^{o}C for 3 h (Ito, 1997). *Bacillus circulans* cellulase was stable at pH from 4 to 10 for 24 h at 30 ^{o}C (Kim, 1995).

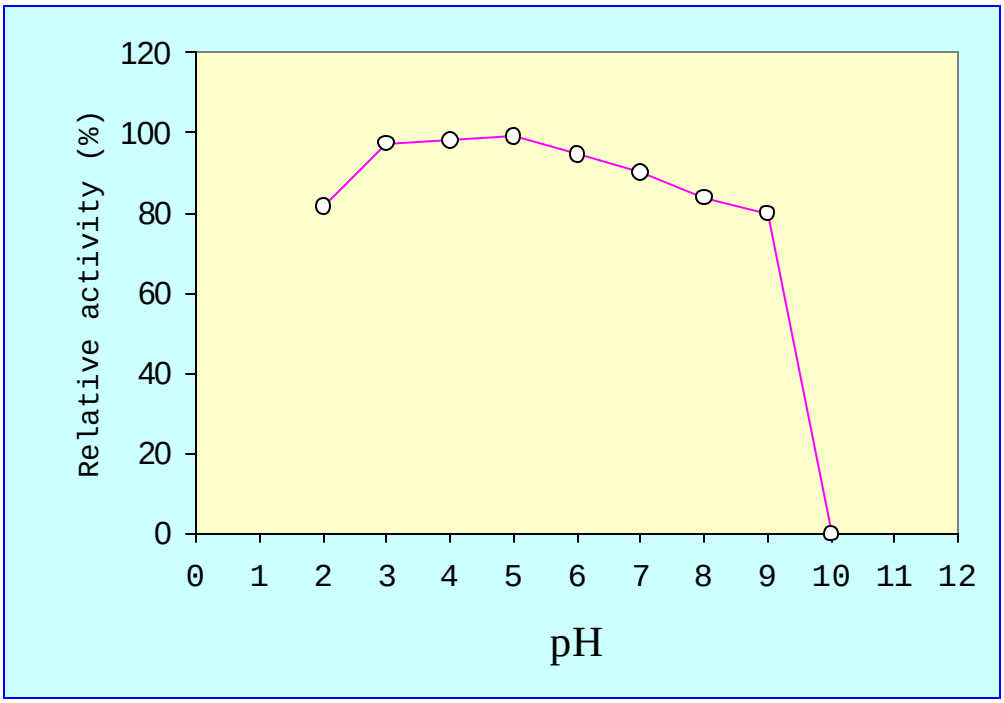

Fig. 28. Effect of pH on cellulase I stability.

5.4.3 Temperature optimum

From the obtained data (Fig. 29) it can be concluded that cellulase I was capable of hydrolyzing the cellulosic substrate at a wide range of temperature from 4 ^{o}C to 60 ^{o}C. This explains why the studied yeast could grow at a broad range of temperature from 5 ^{oC} to 50 ^{o}C. The optimal temperature of the cellulase I (40 ^{o}C) is 10 ^{o}C higher than the optimal growth temperature of the yeast (30 ^{o}C). The enzyme is completely inactivated at 10 ^{o}C higher than maximum temperature of the yeast growth. The temperature profile of the studied yeast correlated with its enzyme temperature range profile. This behavior is almost similar to that of the yeast *Rhodotorula glutinis*, which can grow at a temperature range from 4^{o} C up to below 30 ^{o}C. The optimal temperature for growth was 20 ^{o}C, while its cellulase showed activity at a temperature rang from 4 ^{o}C to 70 ^{o}C with an optimum at 50 ^{o}C (Oikawa et al., 1998). Compared with other cellulases, it was found that the optimum temperature of cellulase activities varied according to the organism. For example, cellulases from *Bacillus* sp. KSM-635 had an optimal activity at 40 ^{o}C, similar to the cellulase in this study (Ito, 1997). While the cellulases from thermophilic organisms had optimal activities at higher temperatures such as 60 ^{o}C for both cellulases from *Bacillus stearothermophilus* and *Clostridium josui* (Kume and Fujio, 1991; Fujino et al., 1989).

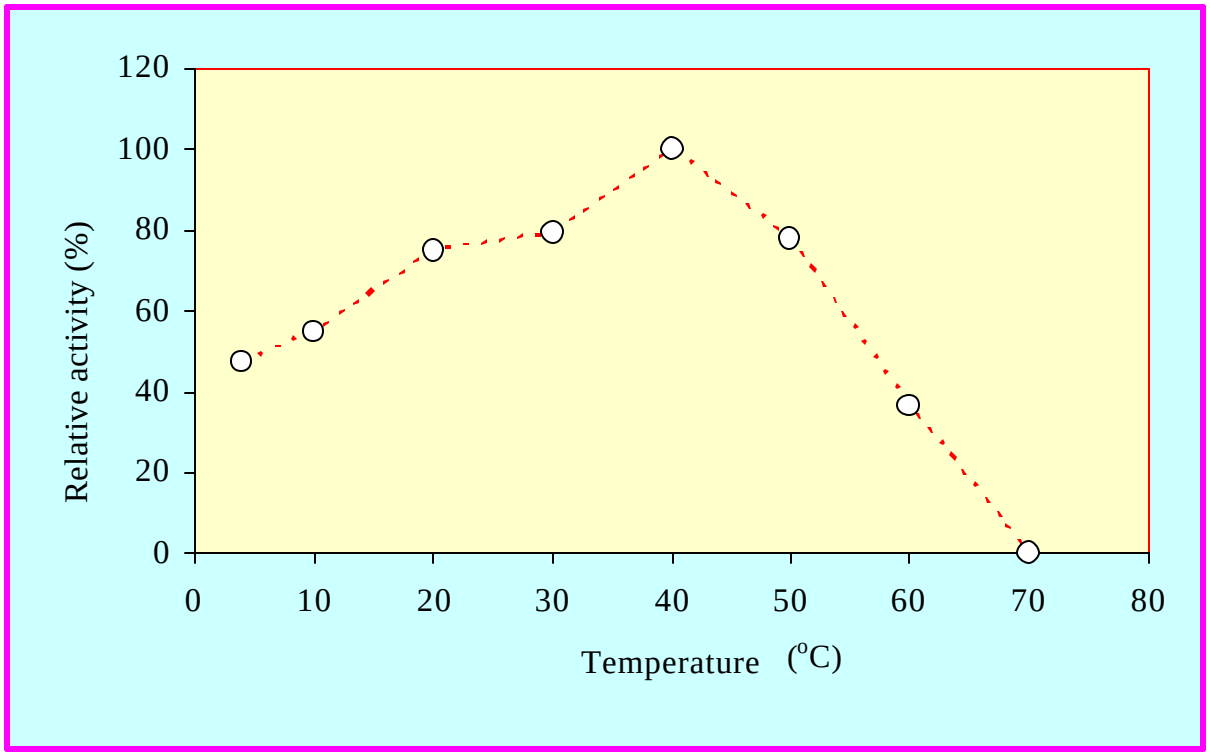

Fig. 29. Effect of temperature on cellulase I activity.

5.4.4 Temperature stability

The enzyme was very stable at -20 °C for 30 months. In addition, it was observed that the enzyme experiences no loss in activity during storage at 4 °C for 48 h. The loss of activity at 40 °C and 50 °C were found to be 4 % and 10 % respectively, but the enzyme was totally inactivated over 50 °C (Fig. 30). The results are to some extent similar to that of the cellulase of the alkalophilic *Bacillus* No1139, which was stable up to 40 °C for 10 min. Ninety % of the original activity was retained at 50 °C and the enzyme was

totally inactived at 60 °C (Fukumori et al., 1985). The studied enzyme was more stable compared to the cellulase from the yeast *Rhodotrula glutinis*, which was found to be stable up to 30 °C, when it was incubated in 20 mM acetate buffer pH 5 for 60 min. More than 25 % of initial activity was lost at 40 °C (Oikwa et al., 1998). The cellulase from *Chalara paradoxa*, which was inactivated at 50 °C in 30 min (Lucas et al., 2001). On the other side, the *Bacillus circulans* cellulase was more stable than the studied enzyme, which was stable up to 50 °C. Seventy-eight % of its activity remained after 72 h, and the enzyme was inactive at 80 °C (Kim, 1995)

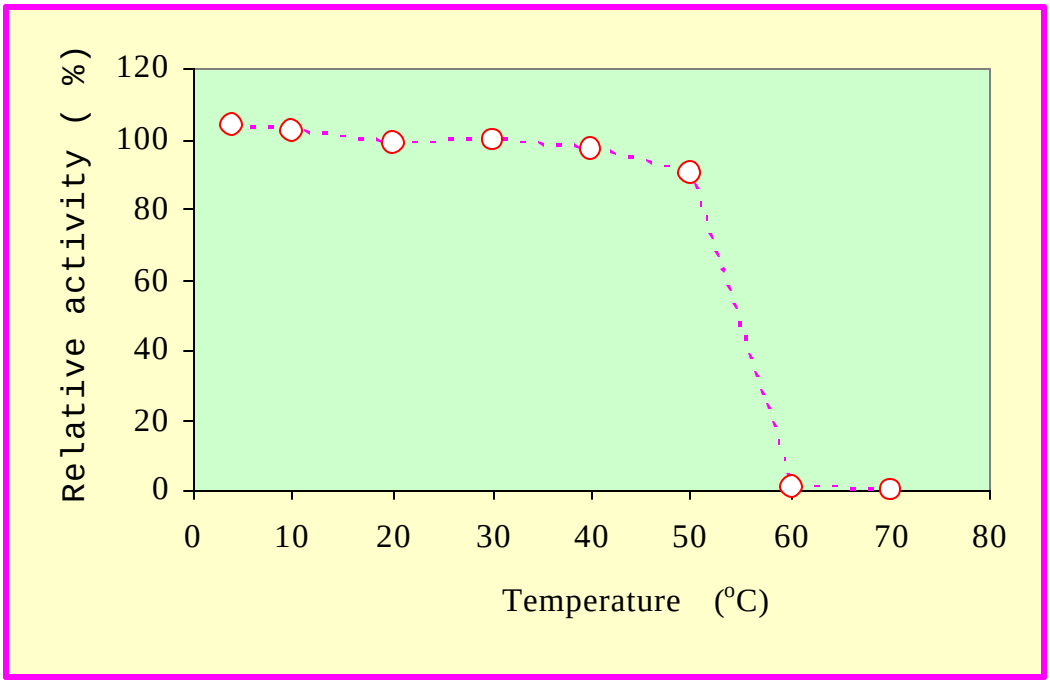

Fig. 30. Effects of temperature on cellulase stability.

5.4.5 Various compounds as activators or inhibitors

The activity of cellulase I was not altered in the presence of surfactants (Fig. 31) tween 40, tween 80, pluronicF68 and silicone antifoam. These results may suggest that these detergents at the studied concentration probably do not play a role on enzyme activity. Even though, the presence of surfactants in growth media enhanced the release of cellulases. These results are in disagreement with the observation by others (Wu and Ju, 1998). They proved that pluronic F68 and F88 as well as tween 20 and 80 enhanced the enzymatic hydrolysis of pretreated newsprint using *Trichoderma reesei* cellulase. On the other hand, the activity of cellulase (I) was reduced by 25 % of its original activity in the presence of triton X-100. These findings were supported by data on *Chalara paradoxa* (Lucas et al., 2001).The effect of reducing agents on cellulase I activity was dependent on the type, nature of enzyme substrate (Mackenzie and Bilous, 1982) and the reducing potential and the nature of the agents. Highest inhibition was observed with 2-mercaptoethanol a final concentration of 0.1%. These results differ from those reported by Johnson et al., (1982), which revealed that cysteine, glutathione and mercaptoethanol had no effect on the solubilization of phosphoric acid-swollen avicel or trinitrophenylcarboxymethylcellulose by cellulase from *Clostridium thermocellum*. In addition, they stated that reducing reagents had negligibly effect on endoglucanase activity from *Clostridium josui* (Fujino et al., 1989). In other reports, reducing agents

significantly enhanced cellulose-solubilizing activity from *Acetivibrio cellulolyticus* (Mackenzie and Bilous, 1982).

Data obtained from this study also revealed that EDTA as a chelating agent had no effect on the activity. This may rule out that cations are not involved in active catalytic site of the enzyme. Similar results were reported by Ng and Zeikus (1981). Their observation showed that EDTA had no effect on the activity of cellulase from *Clostridium thermocellum*. Also ß-glucosidase from *Aspergillus oryzae* and *Candida peltata* were not affected by 10 mM EDTA (Riou et al., 1998; Saha and Bothast, 1996).

Strong inhibition of cellulase I was observed with SDS at 0.1% final concentration. Similar results were obtained by 10 mM SDS on endoglucanase from *Bacillus circulans* (Kim, 1995).

Cellulase from *Chalara paradoxa* was inhibited by Triton X-100, Tween 80 and SDS, but DTT, 2-mercaptoethanol and cystine did not inhibit the activity (Lucas et al ., 2001). The purified ß-glucosidase from the above organism was inhibited by detergents such as SDS, Tween 80 and Triton X-100, but was not inhibited by DTT, 2-mercaptoethanol and cysteine, (Lucas et al., 2000). Dithiothreitol and SDS at a concentration of 5 mM reduced the activity of purified ß -glucosidase from *Bacillus polymyxa* by 30 % and 85 %, respectively (Painbeni et al., 1992). The chelating agent EDTA did not inhibit the purified ß-glucosidase from *Aspergillus oryzae*, but the activity was significantly inactivated by SDS and N-bromosuccinimide (Riou et al., 1998).

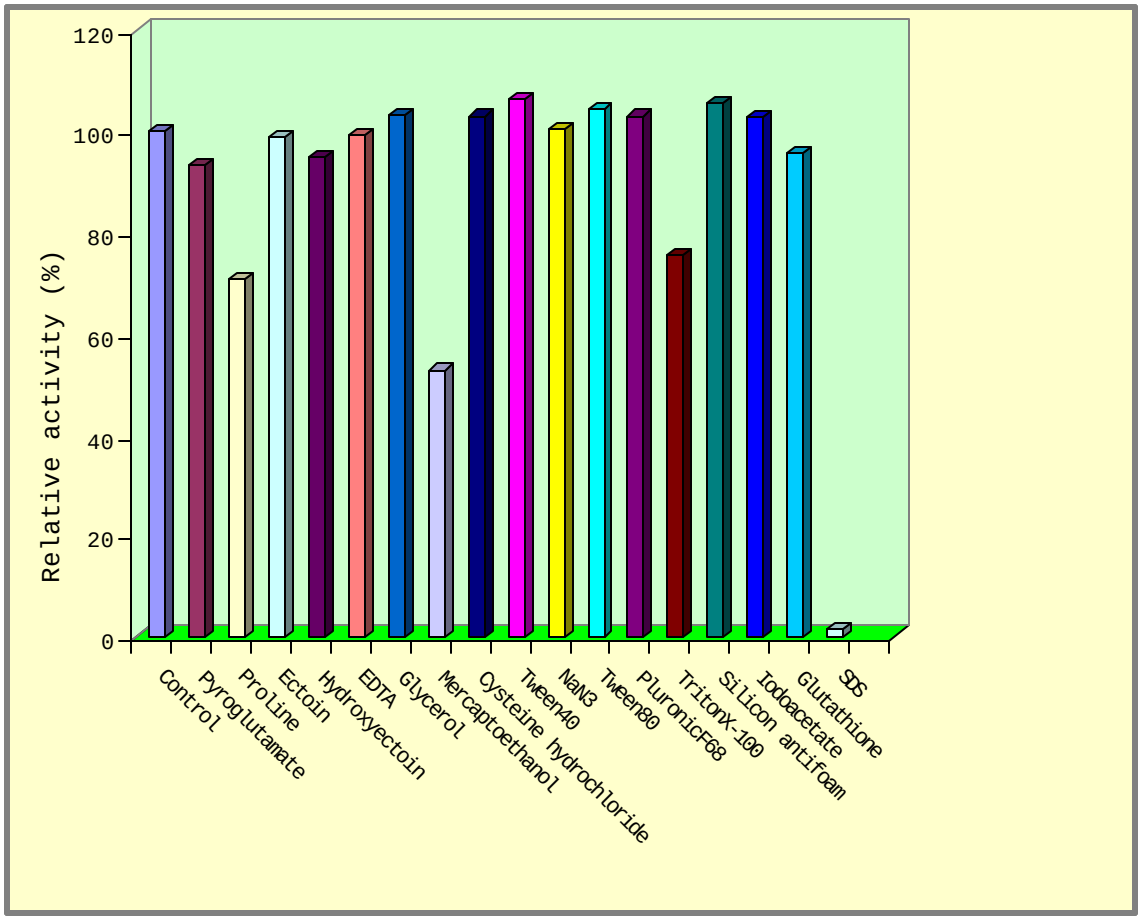

Fig. 31. Effects of various chemicals on cellulase I activity.

5.4.6 Metal ions as activator or inhibitor

The obtained results (Fig. 32) revealed that a stimulating effect on the cellulase activity was caused by Cu^{++} and Mn^{++}.

In accordance, Mn^{++} stimulated the activity of cellulases from *Chalara paradoxa* and *Acetivibrio cellulolyticus* (Lucas et al., 2001; Mackenzie and Bilous, 1982) respectively.

However, variable inhibition on the activity was found by the heavy metals Pb^{++}, Cd^{++}, Ag^{+} and Hg^{++}.

The inhibition by sulfhydryl oxidant metals (Ag^{+} and Hg^{++}) may indicate that the thiol groups are involved in the active catalytic site. The inhibition by reducing agents such as mercaptoethanol also supported this assumption. On the other hand, divalent cations such as Cu^{++} and Mn^{++} stimulated the activity whereas they also can bind on thiol groups, when it is (thiol group) located in the active site. From the former observations, it can be concluded that sulfhydryl groups may not be involved in the catalytic center of the enzyme but rather may be essential for maintenance of the three dimensional structure of the active protein (Riou et al., 1998; Rutter and Daniel , 1993).

On the other side the no effect of EDTA on the cellulase I activity indicated that divalent cations are not required for enzyme activation. However, both Cu^{++} and Mn^{++} stimulated the cellulase I activity. Since they are not involved in the stability of the enzyme, these cations could play a role in the enzyme function, e.g. by modulating its activity according to environmental conditions (Riou et al., 1998). The cellulases enzymes vary in their response to different metal ions. Cellulase from *Bacillus* sp. No. 1139 was stimulated by addition of Na^{+} or K^{+} but completely inhibited by Hg^{+} or Cd^{++} (Fukumori et al., 1985). In other study, the cellulases activity from

Bacillus stearothermophilus was stimulated by Na+ and Ca++ and inhibited by Hg++ (Kume and Fujio, 1991).

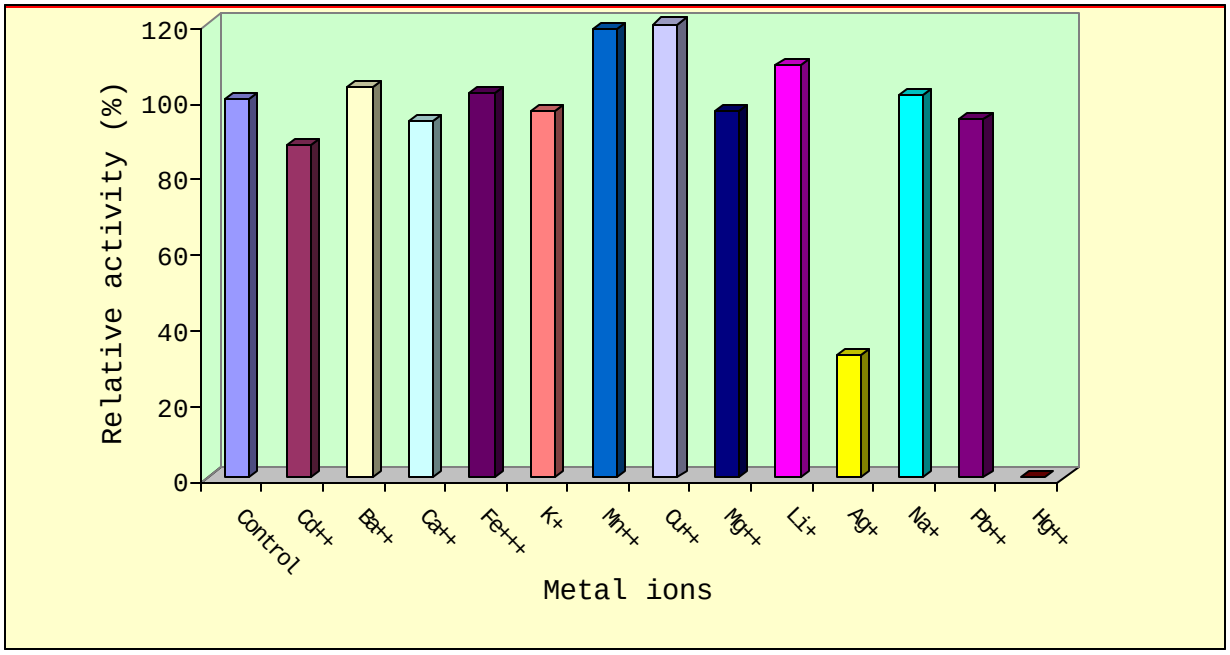

Fig. 32. Effects of metal ions on cellulase I activity.

5.4.7 Inhibition by organic solvents

In general, all tested organic solvents (Fig. 33) did not stimulate the hydrolytic activity of the cellulase I for hydrolysis of CMC. Organic solvents showed a different degree of inhibition to the enzyme. Both ethanol and acetonitrile completely inhibited the hydrolytic activity. Ethylenglycol, methanol and dimethylsulfoxid suppressed the hydrolytic action by 50 % of

the original activity. Although the scarce information available on the effects of organic solvents. It can be suggested that the inhibition effect of tested organic solvents is most likely due to their denaturation effect on the enzyme protein. These results are in accordance with those by Lucas et al., (2001). They found that organic solvents (methanol, ethanol, acetonitrile, ethyl acetate and dimethylsulfoxide) caused inhibition of the activity of cellulase from *Chalara paradoxa* at different degrees.

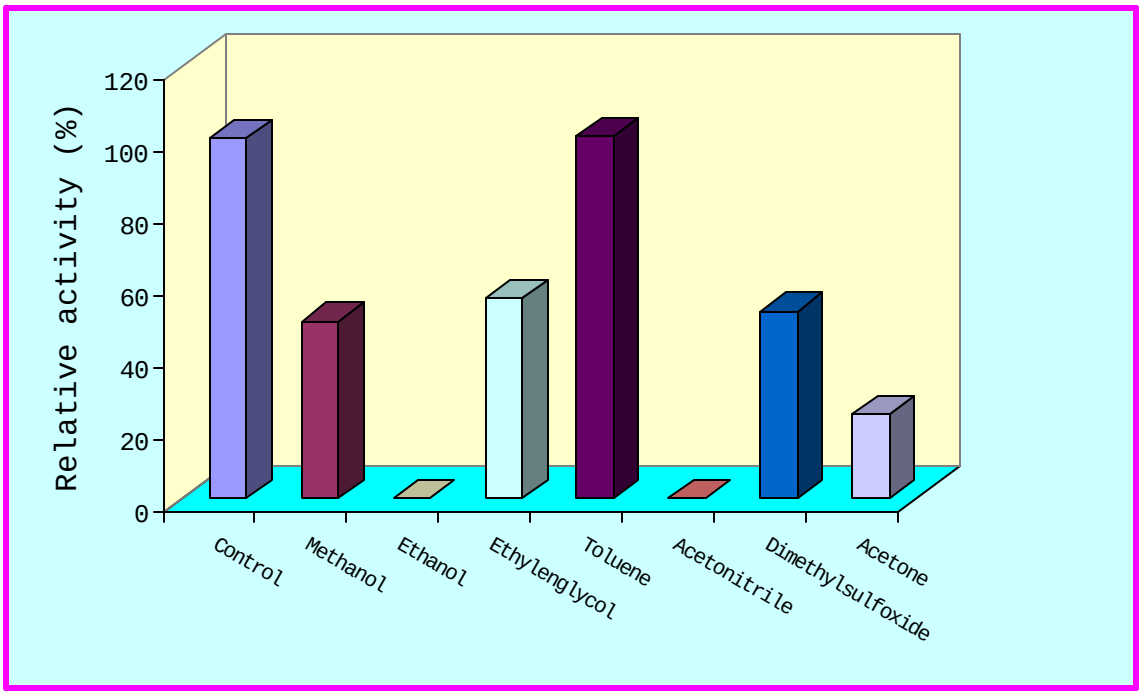

Fig. 33. Effects of organic solvents on cellulase I activity.

5.4.8 Substrate specificity

The purified cellulase I had relatively high substrate specificity. It can tolerate a variety of cellulosic substrates. Its specific activity was dependent on the characteristics of the cellulosic material. CMC was the favorite substrate, which was hydrolyzed with 36 U/mg. Also the specific activity of the enzyme against acid swollen avicel was higher than that of sigmacell, whereas the treatment with phosphoric acid broke the hydrogen bonds between oxygen of alternating glycosidic bonds in one glucan chain and the primary hydroxyl groups at position 6 of glycosyl residues in another chain (Wood et al., 1995). Data also pointed out that the lowest specific activity was found with cellulose powder. These may be due to the production of an irreversible tight complex between cellulase and amorphous cellulose (Carrard et al., 2000). The significant specific activity of cellulase I towards xylan explained that the enzyme has a flexible specificity for the C-6 position of the glucopyranosyl unit of cellulose. On the other hand, the enzyme showed no ability to attack the tested di- and trisaccharides with different ß -linkages. This indicated that all ß-glucosidase enzymes that are required for hydrolysis of these saccharides were separated efficiently during the purification process. In comparison of the obtained results with exo-1,4-ß-glucanase (Avicelase II) from *Clostridium stercorarium*. It can be observed that both enzymes were similar in displaying activity towards microcrystalline cellulose, increase the activity by acid treatment of microcrystalline cellulose, exhibiting activity towards xylan and showed no activity towards p-nitrophenyl-ß-derivatives. However, the studied cellulase

had high specificity towards CMC, while avicellase II had no activity towards CMC (Bronnenmeier et al., 1991). Results also are in accordance with those of endo-1,4 ß-glucanase from *Clostridium josui* which hydrolyzed significantly microcrystalline cellulose avicel but the extent of hydrolysis was remarkably lower than that of CMC (Fujino et al., 1989).

5.5 Mode of action and synergism of cellulases

HPLC results demonstrated that the enzyme was capable to degrade the cellulosic material and its derivatives to ß-(1,4)oligosaccharides with different chain length. Avicel or CMC were degraded to oligosaccharides cellobiose; cellotriose; cellotetraose; cellopentaose at a molar ratio; 32 : 16 : 8 : 1, respectively. On the other hand, glucose was not found among the degradation products. From the degradation products and their molar ratio, the degradation behavior of the used enzyme can be supposed as follows:

The enzyme hydrolyzed the cellulosic substrates to cellopentaose and cellotetraose, and then cleaved the cellopentaose to cellobiose and cellotriose. It splits also the cellotetraose into two units of cellobiose. However, it could not attack both cellobiose and cellotriose therefore; glucose was not detected among the hydrolysis products. Similar behavior was reported from the endo-ß-1,4-glucanase I (Avicelase I) from *Bacillus circulans* (Kim, 1995) and endo-ß-1,4-glucanase from *Clostridium josui* (Fujino et al., 1989). These reported data supported our supposition. Whenever the cellulase reaction was supplemented with ß-glucosidase from

the same yeast, the detected products were only glucose and cellobiose in a molar ratio of 10:1, respectively. These results showed that Cellulase I had a synergistic interaction with ß-glucosidase from the same yeast to complete hydrolysis of the cellulosic materials up to glucose units. Both enzymes work together as a complete enzyme system, in a sequential manner.

5.6 Systematic position of the yeast isolate.

The systematic position of the yeast isolate PAG1 was determined by 18S rDNA sequence analysis. It showed a close relationship to the described species *Trichosporon*. The complete identification of the isolated yeast and its assignment to one species requires physiological and morphological tests in addition to molecular methods of DNA analysis.

6 SUMMARY

Cellulose is the most abundant organic biopolymer on earth. It is a linear polysaccharide of glucose residues connected by ß-1,4 linkages. Effective utilization of cellulosic material through bioprocesses will be an important key to overcome the shortage of foods, feed and fuels, which the world may face in the near future because of the explosive increase in human population. Therefore, cellulose degrading enzymes stimulated our interest to conduct an extensive study on new cellulase sources from different perspectives. This work was aimed to isolate, screen a wild type strain of a cellulolytic yeast and study the suitability of its cellulases for bioprocesses. The isolated yeast was partially identified by using PCR. It showed 100 % sequence identity with *Trichosporon japonicum, T. asahii, T. aquatile, T. faecale, T. coremiiforme, T. aquatile* and *T. asteroids.* The complete identification of the isolated yeast and its assignment to one species requires physiological and morphological tests in addition to molecular methods of DNA analysis.

Nutritional and environmental factors which were extensively studied to monitor the growth and cellulase production. The isolated strain showed growth and cellulase production at a broad range of temperature from 5 ^{o}C to 50 ^{o}C with an optimal cellulase production at 30 ^{o}C.

Different cellulosic materials and oligosaccharides stimulated the cellulase excretion, but the best induction was exhibited by lactose 1 % w/v.

The organic nitrogen compounds stimulated higher cellulase yield than inorganic sources. Peptone at concentration between 0.5 and 0.8 (w/v) induced the best yield. The yeast survived in media of pH values from 3 to 10. At pH 7 the optimal growth and cellulase production was observed. Surfactants enhanced the release of cellulase. Highest yield was obtained in the presence of 0.5 % Tween 80. The cellulase production was induced by some oligosaccharides but the best induction was exhibited by lactose 1 % w/v. Culture agitation improved the cellulase excretion; maximum release of cellulase was noticed at 400 rpm. Cellulase was detected in the cultivation medium, after 6 h and remained up to more than 72 h of cultivation. The optimum yield was found at 24 h. Enzymes purification was carried out using FPLC technique.

Two cellulase peaks (Cellulase I and cellulase II) were found, but only cellulase I was obtained in a pure preparation. The purified cellulase I was active over a broad pH range from pH 3 to 9. The highest activity was found at pH 5.

The enzyme was stable in a broad pH range; it retained more than 80 % of its normal activity after incubation at pH values from 2 to 9. The enzyme was also active over a broad range of temperatures from 4 ^{o}C to 60 ^{o}C). The temperature optimum was at 40 ^{o}C. The enzyme was thermal stable. It retained more than 90 % of its activity after incubation at 50 ^{o}C. No loss of the enzyme activity was observed during the incubation at 4 ^{o}C for 48 h. The enzyme retained full activity after storage at -20 ^{o}C for 30 months. The effects of chemical compounds on the enzyme activity were determined. The tested surfactants showed no effect on the activity, except Triton X-100

which reduced 25 % of the original activity. Most of the tested reducing agents had no effect or showed slightly inhibition on the activity except 2-mercaptoethanol which reduced 50 % of the optimal activity. On the other hand, SDS was the strongest inhibitor. The enzyme was inhibited by the classic metal ion inhibitors such as $AgNO_3$ and $HgCl_2$. In contrary $CuCl_2$ and $MnCl_2$ stimulated the enzyme activity. Organic solvent caused variable degrees of inhibition. Inhibition effect on the enzyme activity with different potential was observed in case of lactose, cellobiose, maltose and gentiobiose. Total inhibition occurred at 2 mM lactose, while both cellobiose and maltose caused complete inhibition at 2.6 mM. The purified cellulase I showed activity towards many types of cellulosic materials such as CMC, sigmacell, xylan, cellulose powder and acid swollen avicel, while it did not show any activity towards the different types of the tested oligosaccharides and p-nitrophenyl derivatives. HPLC analysis of the degradation products demonstrated that the enzyme was capable to degrade the cellulosic material and its derivatives to ß-(1,4) oligosaccharides with different chain length. Avicel or CMC was degraded to the oligosaccharides cellobiose; cellotriose; cellotetraose and cello- pentaose, at a molar ratio; 32:16:8:1, respectively. On the other hand, glucose was not found among the degradation products. Whenever the cellulase reaction was supplemented with ß-glucosidase from the same yeast, the detected products were only glucose and cellobiose in a molar ratio of 10:1 respectively.

7 Abstract

Production, Purification, Properties and Application of the Cellulases from a Wild type Strain of a Yeast isolate.

The effective and economic utilization of cellulosic materials will be an important means to overcome the shortage of foods, feed and fuels, which the world may face in the near future. Therefore, we have performed intensive investigations on cellulases from newly isolated yeast strain. The cellulase producing capability of one yeast strain from the soldier bug *Pyrrhocoris apterus* was studied in more detail. The systematic position of the yeast isolate PAG1 was determined by 18S rDNA sequence analysis. It showed a close relationship to the described species *Trichosporon*. The growth conditions for optimal cellulase production were studied. One of the produced cellulases was purified to homogeneity. Its biochemical characteristics, e.g. substrate specificity, temperature and pH optimal as well as the influence of chemical compounds, were determined. Analysis of the degradation products demonstrated that crystalline cellulose and carboxy-methylcellulose were degraded to cellobiose, cellotriose, cellotetraose and cellopentaose in a molar ratio of 32:16:8:1, respectively. When ß-glucosidase from the same yeast strain was added only glucose and cellobiose in a molar ratio of 10:1 were detected. Only one report on cellulase production by yeast strains has been published so far. Our investigations show for the second time that also wild type yeast strains can produce cellulases with some interesting features.

8 Kurzzusammenfassung

Produktion, Reinigung, Eigenschaften und Anwendung von Cellulasen eines Wildtyp-Hefeisolates.

Die effiziente Verwendung von Cellulose wird in naher Zukonft ein wichtiges Instrument zur Vermeidung einer Nahrungsmittel- und Energieknappheit werden. Deshalb haben wir uns intensiv mit Cellulasen befaßt, die aus Hefestämmen isoliert wurden. Die Fähigkeit der Cellulase-produktion eines Hefe-Stammes der Feuerwanze *Pyrrhocoris apterus* wurde genauer untersucht. Die systematische Stellung des Hefe-Isolates PAG1 wurde durch Sequenzierung der 18S rDNA bestimmt. Es zeigte eine nahe Verwandtschaft zu einem bereits beschriebenen Stämme der Gattung *Trichosporon*. Außerdem wurden die Wachstums-bedingungen für eine optimale Cellulase–Produktion bestimmt. Anschließend konnte eine der produzierten Cellulasen mit FPLC aufgereinigt und deren biochemische Eigenschaften (z.B. Substratspezifität, Temperatur optimum, optimaler pH-Wert, Einfluß von Chemikalien) untersucht werden. Eine Analyse der Abbau-Produkte zeigte, daß kristalline Cellulose und CMC zu Cellobiose, Cellulotriose, Cellulotetraose und Cellulopentaose in einem molaren Verhältnis von 32:16:8:1 umgesetezt wurden. Bei Zusatz von ß-Glykosidase aus demselben Hefestamm entstand nur Glucose und Cellobiose in einem molaren Verhältnis von 1:10. Da bisher nur eine Publikation über Cellulase-produzierende Hefe-Stämme erschienen ist, zeigen auch unsere Untersuchungen, daß Wildtyp-Hefestämme Cellulasen mit interessanten Eigenschaften produzieren können.

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