2. Regulation of OXDC

2.1 Introduction

A decade ago, the idea that, the transcriptional machinery from organisms as divergent as *Drosophila*, human and yeast could largely be conserved seemed unbelievable. Subsequently, much of the transcription initiation complex has been found to be highly conserved both structurally and functionally between these organisms. The idea that there might be significant differences in the way transcription is fine-tuned in different organisms has almost become taboo. As more detailed knowledge of different transcription systems have become available, however, the pendulum of opinion appears to be swinging towards the notion that there may be some interesting and potentially significant differences between the mechanisms regulating transcription in different organisms. *Collybia velutipes* is a basidiomycetous, saprophytic edible fungus, popularly known a “Increasing Intelligent mushroom.” It’s biology yet has not been explored in detail. Earlier, gene for an oxalate catabolising enzyme, oxalate decarboxylase was cloned from this organism (Mehta and Datta, 1991). To study of the regulation of OXDC gene and mechanisms involve in transcriptional activation; it was of interest to isolate the upstream region of this gene. Once the sequence is available, the regulation of expression can be studied. To achieve this a 2.5 kb upstream region of OXDC was isolated and subcloned into pTZ18U and named as pSC24.

2.2 Materials and methods

2.2.1 Biological materials

*Collybia velutipes* (Strains, ATCC 13547)
*Escherichia coli* [LE392, DH5 α, BL21 (DE3)]
*Saccharomyces cerevisiae* [BJ6485, KY114, AH22]
*Schizosaccharomyces pombe* (BJ 7468)

2.2.2 Media and solutions

All % shown are on W/V basis (unless mentioned otherwise)

All restriction enzymatic manipulations were performed by the supplier’s instruction.

**LB:** 1% Tryptone, 1% Sodium chloride, 0.5% yeast extract; pH adjusted to 7.5 by NaOH.

**LB Agar:** LB with 1.5 % agar.

**2XL:** 2% tryptone, 1% yeast extract and 0.1% Sodium chloride; after autoclaving 0.2% Glucose was added.

**Media with ampicillin:** LB or LB agar whenever indicated contained 50µg ampicillin/ml of medium
**Media with ampicillin:** LB or LB agar whenever indicated contained 50μg ampicillin/ml of medium.

**TB (Terrific broth):** Tartof and Hobbs, 1987
Bacto tryptone 1.2%, yeast extract 2.4% glycerol 0.4% was autoclaved and then after autoclaving 10ml phosphate buffer was added (0.17M, KH₂PO₄ 0.72M K₂HPO₄) which was autoclaved and stored at room temp.)

**YPD:** 1% yeast extract, 2% peptone, 2% dextrose

**YPD Agar:** YPD with 1.5% agar

**SD:** 0.67% Yeast nitrogen base without amino-acid; 2% dextrose, supplemented with different amino acids at concentration of 20-30mg/litre.

**SD Agar:** SD with 1.5% agar

**Medium for Collybia:** 5% dextrose, 1% peptone, 0.1% KH₂PO₄, 0.05% MgSO₄.7H₂O and 1% Difco malt extract (pH 5.2)

**EndoR:** 6 x =30% F1Coll 400, 60mMEDTA pH 8.0, 0.6% SDS and 0.06% bromophenol blue.

**Phenol:** Glaxo laboratories Excel AR grade was redistilled at 180°C and stored frozen at -20°C in small aliquots.

**Laemmlli buffer:** 3X= 0.1875M Tris-Cl pH 6.8, 15% (v/v) β-mercaptoethanol, 6% SDS, 30% sucrose, 0.006% bromophenol blue

**20X SSC:** 175.3g NaCl and 88.2g trisodium citrate were dissolved in 800ml MQ water. The pH was adjusted to 7.0 by adding 200μl of 50% HCl, volume was made up to 1 liter and sterilized by autoclaving.

**RNase A:** 10mg/ml. Stock was prepared as described in Sambrook *et al.*, (1989).

### 2.2.3 Growth and cultivation of Collybia velutipes

*Collybia* was grown on the surface of the medium. The organism was grown from mycelial inoculation at 25°C in stationary cultures. About 25 days after inoculation, oxalate decarboxylase was induced by addition of oxalic acid to 12.5 mM, potassium oxalate to 12.5mM and HCl 11.4mM. The mycelium was harvested 2hr-post induction, Mycelial pad was washed with cold autoclaved water, and stored at -20°C. *C. velutipes* was maintained on slants of the same medium (Jakoby, 1962).

### 2.2.4 Isolation of total RNA

RNA was isolated by the method of Chomczynski and Sacchi, (1987) with some modifications. The original method designed for animal cells was modified for fungal cells.

1. The vegetative mycelium of *C. velutipes* was frozen at -20°C and ground in liquid nitrogen. The powder was transferred into preweighed capped polypropylene tube.
2. 2.7 ml of filter sterilized 1.25 X solution D (5M Guanidine thiocyanate 31.25mM Sodium citrate pH 7.0, 0.625% w/v sarcosyl, 0.125M β-mercaptoethanol) and 3.5g of 0.45mm glass beads were added per gram net weight of Collybia velutipes.

3. The suspension was vortexed at full speed for three minutes in Biospec homogenizer.

4. The following solution were sequentially added thereafter:
   (i) 2M sodium acetate, pH (4.0) 0.340ml.
   (ii) Water saturated phenol 3.4ml.
   (iii) Chloroform: Isomylalcohol (24:1) 0.7ml.

   It was thoroughly mixed and incubated on ice for 15min.

5. The mixture was centrifuged at 10,000g for 20 min at 4°C, and from the aqueous phase, total RNA was precipitated with equal volume of isopropanol at 4°C for 30 min.

6. RNA was pelleted at 10,000g for 20 min at 4°C and the pellet was dissolved in 1ml of 1X solution D. It was then heated to 60°C for 15 min to assist dissolution.

7. RNA was reprecipitated with equal volume of isopropanol.

8. The pellet was resuspended in 1ml of 3M sodium acetate (pH5.5.), incubated on ice for 10 min. and spun at 10,000g for 10 min at 4°C to remove any DNA contamination. This step was repeated twice and the pellet was washed twice in 75% ethanol, dried at room temperature and resuspended in DEPC treated water. Total RNA obtained was quantitated spectrophotometrically at absorbance 260 and 280nm.

2.2.5 Agarose Gel electrophoresis of RNA and Northern blotting

Formaldehyde-denatured RNA gel electrophoresis (Lehrach et al., 1977) was performed as described by Sambrook et al. (1989) using formaldehyde (Glaxo) pH 3-3.5. Formamide was deionized and stored in small aliquots at -20°C.

1. RNA samples (30μg) 4μl
   10XMOPS 2μl
   Formamide 10μl
   Formaldehyde 3.5μl

   Mixed and incubated at 60°C for 15 min and then chilled on ice.

2. 2μl loading buffer (50% Glycerol, 1mMEDTA, 0.002% Bromophenol Blue and 0.002% Xylenecyanol) was added.

3. 1.5% (W/V) agarose gel containing 1XMOPS and 2.2 M formaldehyde was prepared.

4. Gel was placed in electrophoresis tank with 1 X MOPS buffer and was prerun for 15 min.

5. Samples were loaded, and was run at 80V (about 3-4hrs).

6. After electrophoresis, gel was rinsed thrice in DEPC treated water, followed by RNA hydrolysis by soaking in 50mM NaOH, 10mM NaCl for 45min.
7. The gel was then neutralized in 0.1M Tris-Cl pH 7.5 and RNA transferred to gene screen plus membrane using 10XSSC as described (Gene Screen Plus protocols, NEN), and baked for 2 hr at 80°C to reverse formaldehyde reaction.

8. The amount of RNA loaded in the gel was estimated by ethidium bromide staining (Sambrook et al., 1989) of parallel lanes.

9. The mobility of formaldehyde treated DNA is not comparable to that of RNA. In absence of RNA standards, size of RNA was estimated using glyoxal system. However, one of the advantages of formaldehyde system is that the transfer is more efficient and consequently transfer time is short (3-4h).

**2.2.6 Nucleic Acid hybridization**

Northern hybridization was performed as suggested by the supplier of the Gene Screen Plus membrane (NEN Research products, DuPONT).

1. Blots were prehybridized (1MNaCl, 50% formamide, 10% dextran sulphate, 1% SDS) for 6-8 hr. at 42°C in hybridization incubator.

2. Probe was prepared by random primer labelling method, using the NE Blot Kit,

3. Probe was combined with sheared Salmon sperm DNA (100µg/ml hybridization solution), denatured at 95°C for 10min and chilled immediately.

4. Probe-DNA was then added into prehybridization solution mixed and incubated at 42°C for 12-24hr.

5. Blot were washed thrice with 2XSSC at room temperature for 10 min

6. Washed thrice in 2X SSC, 1% (W/V) SDS at 65°C for 15 min.

7. Blots were then washed in 0.1XSSC with three changes for 15 min at room temperature.

8. Blots were sealed in plastic bags and exposed to Amersham Hyper film at -70°C for 24-36h.

9 Film was developed.

**2.2.7 Primer extension**

**2.2.7.1 Labeling of Oligo**

1. The reaction mixture was prepared as follows.
   
   10pmol/µl Oligo 06
   
   10X polynucleotide Kinase buffer
   
   10U/µl T4 polynucleotide Kinase2µl.
   
   10µCi/µl [γ-32 P] ATP
   
   Milli-Q H$_2$O

   Mixed thoroughly and incubated at 37°C for 1h.

2. Reaction was stopped by heat inactivation at 65°C for 20 min.

3. The labeled primer was purified by passing through Sephadex G-50, 1ml column. as described in Sambrook et al., (1989)
4. Radiolabeled oligonucleotide was precipitated by adding 0.5 volume ammonium acetate and 2.5 volume of ethanol & incubated at -70°C for 20 min.

5. Samples were centrifuged at 12000g at RT supernatant was discarded and pellet was washed twice with chilled 70% ethanol.

6. Pellet was vacuum dried and count was taken in Pharmacia counter.

7. The pellet was resuspended in Milli-Q water to get the final concentration was 20,000cpm/µl.

2.2.7.2 Hybridization of radiolabeled oligo to RNA

1. 10µg RNA and 40,000 Cpm (Oligo) was mixed in 1X Reverse transcriptase buffer.

2. Samples were incubated on 65°C water bath for 60 min

3. After 60 min samples were removed and cooled slowly to room temperature.

2.2.7.3 Extension

i By Superscript II

1. Reaction was started by adding dNTPS upto 300µM and 400 units of M-Mulv Reverse transcriptase (Superscript II GIBCO BRL).

2. Reaction mix was incubated at 42°C for 40-60 min.

ii Primer extension reaction by rTTh

\[
\begin{align*}
&5 \times \text{EZ buffer} & 10µl (1X) \\
&\text{dNTPS} & 6µl (250µM) \\
&Mn(OAc)_{2} & 5µl (2.5mM) \\
&06 & 2µl (40,000cpm) \\
&r\text{TthDNA polymerase} & 2µl (5U/50µl) \\
&\text{RNA} & 1µl (1µg) \\
&H2O & 24µl
\end{align*}
\]

Reaction mix was incubated at 60°C for 1 hr.

1. Reaction sample was taken out and precipitated by 0.5 volume of ammonium acetate and 2 volume of ethanol.

2. The DNA was pelleted by centrifugation and washed twice by 70% ethanol.

3. The samples were resuspended in 10µl gel loading buffer (90% Formamide, 0.002% bromophenol blue and 0.002% Xylenecyanol).

4. Samples were incubated at 90°C for 5 min and chilled on ice.

5. Whole sample was loaded on 8% acrylamide-urea gel. Simultaneously a sequencing reaction was carried out with the same primer using circumvent DNA sequencing kit (NEB) according to supplier's instruction and loaded.
2.2.8 Subcloning of oxalate decarboxylase upstream regions:

2.2.8.1 Polymerase chain reaction

Design of oligo for use in PCR (Saiki et al., 1988). The Oligo corresponding to -533 to -542 (03) position with respect to ATG was synthesized. The down-stream oligo 012 for amplifying initial 500-bp promoter region was generated with a Bgl II site which spans the ATG to facilitate translational fusion with β-galactosidase in pSZ211. 03 and 012 were designed using PCR PLAN of PC-GENE software.

Amplification of OXDC promoter by (012 and 03) reaction was performed in 100μl reaction. Reaction mix was prepared by mixing the following reagents.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milli-Q H₂O</td>
<td>48.5μl</td>
</tr>
<tr>
<td>10XPCR buffer</td>
<td>10.0μl</td>
</tr>
<tr>
<td>dATP 10mm</td>
<td>2.0μl</td>
</tr>
<tr>
<td>dGTP</td>
<td>2.0μl</td>
</tr>
<tr>
<td>dCTP</td>
<td>2.0μl</td>
</tr>
<tr>
<td>dTTP</td>
<td>2.0μl</td>
</tr>
<tr>
<td>Primers</td>
<td></td>
</tr>
<tr>
<td>Oligo 03 5pmol/μl</td>
<td>10.0μl</td>
</tr>
<tr>
<td>Oligo 12 &quot;</td>
<td>10.0μl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.5μl</td>
</tr>
<tr>
<td>MgCl₂ 23mM</td>
<td>8.0μl</td>
</tr>
<tr>
<td>Template (psc24)</td>
<td>5.0μl</td>
</tr>
</tbody>
</table>

100.0μl

The sample was mixed and transferred to thermal cycler (M.J. Research PTC-100). Two step cycle condition was used.

\[
\begin{align*}
94^\circ C & \quad 1\text{ min} \\
40^\circ C & \quad 1\text{ min} \\
72^\circ C & \quad 1\text{ min} \\
94^\circ C & \quad 1\text{ min} \\
52^\circ C & \quad 1\text{ min} \\
72^\circ C & \quad 1\text{ min} \\
72^\circ C & \quad 10\text{ min}
\end{align*}
\]

5 cycles

30 cycles

After completion of PCR 2μl sample was analysed on 1% agarose gel.

2.2.8.2 Cloning of the PCR Product

1. The amplified promoter DNA was purified and concentrated through microcon -30 to remove unreacted oligos and dNTPs.

2. The concentrated DNA was quantified, and digested with Nsi I (NEB), at 37°C for 2hr.
3. Digested DNA was electrophoresed in preparative agarose gel. The band was cut out and purified by using Geneclean II kit (Bio 101) according to manual's instruction.

4. Rest of the upstream region was taken out from pSC24 by Nsi I and Hind III digestion. Twenty microgram of DNA was digested in 50μl reaction at 37°C for 2 hr.

5. The digested DNA was electrophoresed through 1% agarose gel.

6. A 2-kb band was excised from gel and the DNA was purified as mentioned above. pBluescriptll KS was digested with Hind III and Sma I. The vector backbone was purified using Genecleanll kit (Bio 101).

Ligation was setup in a 20μl volume with 50ng vector, 50ng of 2.0kb DNA and 20ng of PCR-product. Ligation was done using T4DNA ligase from NEB-in 1X T4DNA ligase buffer supplied by the manufacturer's. Samples were incubated at 16°C for 16-18 hrs. 10μl of ligation mix was used for transforming DH5α cells by the protocol described in following section

2.2.9 Transformation of E.coli

2.2.9.1 Preparation of DH5α competent cells

DH5α was tested for recA as described in Maniatis et al., (1982). Competent cells were prepared according to the method of Cohen et al., (1973).

1. A preinoculum of DH5α was made in 10ml of 2XL medium by inoculating a single colony of a recA tested strain and growing it overnight at 37°C.

2. 1% of preinoculum was added to 50ml of sterile LB medium, and the culture was grown at 37°C until an A600 of 0.5 was reached.

3. The flask was chilled on ice for 30min. The cells were pelleted in a chilled centrifuge tube for 5 min at 6000rpm at 4°C.

4. The cells were resuspended in 0.1M CaCl₂ and incubated for 1 hr on ice.

5. The "competent" cells were spun down as described earlier. The cells were resuspended in 1/20 of the original volume of ice cold 0.1M CaCl₂. 45% glycerol was added dropwise to a final concentration of 15%.

6. The cells were aliquoted into microfuge tubes on ice and snap frozen by dipping in liquid nitrogen and stored at -70°C until use.

2.2.9.2 Transformation

For each transformation, 100μl of competent cells were used. All the steps were performed under sterile conditions.

1. Competent cells were slowly thawed on ice. Ligation mix was added in a maximum volume of 10μl and the tubes incubated on ice for 1hr.

2. The cells were subjected to heat shock at 42°C in a water bath for 2min. and then stored on ice.
3. 0.9ml of LB medium was added and the cells were allowed to outgrow for 1 hr at 37°C with gentle shaking to allow the expression of the antibiotic resistance gene. Cells not receiving any plasmid DNA, and the uncut vector were used as controls.

4. Transformation efficiency was calculated as the number of transformants/μg DNA and was calculated from the transformation of the uncut vector.

2.2.9.3 Plating of the transformation mix
20μl of 0.1M IPTG (BRL) and 16μl of X-gal (50mg/ml) was spread on LB agar plates containing ampicillin. 50-100μl of transformation mix was plated in each case. The plates were dried and incubated in a 37°C incubator, agar side up until the colonies were apparent (12-16hrs). The plates were stored at 4°C until the blue color deepened and the white colored recombinants became distinct.

2.2.10 Screening and analysis of recombinants
Each recombinant colony was picked up with a sterile toothpick and patched on to a fresh master plate and simultaneously inoculated into 2ml of TB medium containing ampicillin. The cells were grown in liquid culture at 37°C in a shaker and the master plate incubated at 37°C until the patched colonies appeared. Plasmids were isolated by miniprep protocol (see below) and analyzed by restriction digestion.

2.2.10.1 Small scale plasmid isolation
Plasmid was isolated from 1.5ml cultures by boiling method as described in Maniatis et al., (1982).

2.2.11 Medium scale plasmid isolation
Plasmid was isolated from 50ml cultures according to the protocol described in Promega Protocol and applications guide (2nd edition).

1. A single colony from the agar plate was inoculated in 50ml of TB medium containing 50μg/ml amp. The cells were grown overnight in a shaker at 37°C.

2. Cells were spun down at 5000 rpm, 4°C for 15min in SS34 rotor (Sorvall). The cells were resuspended in 5ml lysis buffer (25mM Tris-Cl (pH8.0), 10mM EDTA, 50mM glucose). Lysozyme in 10mM Tris Cl (pH8.0) was added to a final concentration of 10μg/ml and incubated in ice water for 10 min.

3. 10ml of freshly prepared 0.2N NaOH, 1% SDS was added. The lysate was mixed by inversion and incubated in ice for 10min.

4. 7.5ml of 3M NaOAc pH 5.2 was added and mixed thoroughly by inversion. The tube was stored in ice for 20min.

5. The lysate was spun at 15,000g for 15min and the supernatant transferred to a fresh tube.
6. DNase free RNase A was added to a conc. of 20μg/ml and incubated at 37°C for 30 min.

7. The sample was extracted twice with an equal volume of phenol/chloroform (1:1). It was vortexed for one minute, centrifuged at 12,000g for 15min and the aqueous phase recovered into a fresh tube.

8. To the aqueous phase equal volume of chloroform: Isoamylalcohol (24:1) was added, vortexed for one minute and centrifuged as above. The aqueous phase was transferred to a fresh tube.

9. Two volumes of ice cold ethanol were added, mixed and stored at -20°C for 30min. The DNA was recovered by centrifuging at 12,000g, at 4°C for 20 min.

The pellet was dissolved in 320μl of sterile water and 80μl of 4M NaCl was added. 400μl of 13% PEG was added, mixed and incubated on ice water for 1hr. The DNA was then collected by centrifugation at 12,000g for 10min. The pellet was rinsed in 70% ethanol, dried and resuspended in 50μl TE. The average yield was 75-100μg.

2.2.12 Generation of unidirectional nested deletions with Exonuclease III

A unidirectional nested deletion of each of the subclones in opposite orientations was generated by the method of Henikoff (1987).

Plasmid DNA preparations without any nicked molecules were used as a substrate for Exonuclease III deletions.

**Solutions required:**

- 5X Exo III buffer-330mM Tris Cl (pH8.0), 3.3 mM MgCl₂
- 7.4X S1 buffer-0.3M KOAc (pH 4.6), 2.5mM NaCl, 10mM ZnSO₄, 50% glycerol.
- S1 stop mix-0.3M Tris base, 0.05M EDTA
- 2X Klenow buffer-40mM Tris Cl (pH8.0), 0.2Mg Cl₂
- 2X dNTP mix-250uM of dATP, dCTP, dGTP, dTTP
- 5% Ligase buffer-0.25M Tris Cl (pH7.6), 50mM MgCl₂, 5mM ATP, 5mM DTT, 25% (w/v) PEG 8000.

1. 5-10μg of recombinant plasmid was cleaved with Eco RI followed by Pst I. A small part of the reaction mix was analysed on 1% agarose gel to check for completion of digest.

2. The double digested plasmid was extracted with equal volume of TE saturated Phenol/chloroform: isoamylalcohol and then precipitated with equal volumes of ethanol. Care was taken to minimize vortexing of the sample to prevent any nicking of the DNA samples.

3. The pellet was rinsed twice with 70% ethanol, air dried and resuspended in 20μl of sterile water.

4. For 2.3-kb inserts 12 time points of deletions were taken.

5. Microfuge tubes were labelled according to timepoints and placed in ice water.
6. S1 reaction mix was prepared as follows

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4X S1 buffer</td>
<td>15.2μl</td>
</tr>
<tr>
<td>S1 nuclease (5u/μl)</td>
<td>6.75μl</td>
</tr>
<tr>
<td>Sterile water</td>
<td>8.5μl</td>
</tr>
</tbody>
</table>

* Exonuclease III (NEB 100U/μl) 1.5μl

* Added after removing zero time point, i.e. control without enzyme.

7. 2.5μl of Exo III reaction mix was added into S1 mix of the tube marked as no enzyme control.

8. The Exo III reaction tube was prewarmed in a water bath set at 37°C for 10 min.

9. 1.5μl (150U) of Exo III was added and quickly mixed thoroughly while containing the incubation at 37°C.

10. 2.5μl aliquots were removed every 30 seconds with a fresh tip and mixed with 7.5μl of S1 reaction mix.

11. The tubes were given a brief spin and the samples incubated at room temperature for 30 min for S1 nuclease to act.

12. 1μl of S1 stop mix was added per reaction tube, mixed and spun briefly.

13. The tubes were heated at 70°C for 10 min to inactivate S1 nuclease. 2μl of each sample was mixed with 13μl of 1X Endo-R sample buffer. The deletions were analysed on 1% agarose gel containing 1μg/ml EtBr. The starting plasmid and the vector alone were used to compare the extent of deletions. The size of the deleted fragments was estimated using DNA standards.

14. The tubes were preincubated at 37°C for 10 min.

15. Klenow reaction mix was prepared as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Klenow buffer</td>
<td>8μl</td>
</tr>
<tr>
<td>Klenow (Pharmacia 1u/μl)</td>
<td>3μl</td>
</tr>
<tr>
<td>Water</td>
<td>5μl</td>
</tr>
</tbody>
</table>

16. 1μl of Klenow mix was added and the tubes incubated at 37°C for 5 min. Next 1μl of dNTP mix was added. The tubes were spun briefly and incubation at 37°C was continued for 10 more min. The tubes were then chilled on ice.

17. Ligase reaction mix was prepared as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X Ligase buffer</td>
<td>48μl</td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td>1μl</td>
</tr>
<tr>
<td>Sterile water</td>
<td>191μl</td>
</tr>
</tbody>
</table>

18. 40μl of the ligase reaction mix was added per sample and incubated at room temperature for 16 hrs.

19. 10μl of the ligation mix was used to transform DH5α. Deleted subclones were analysed by the mobility of the supercoiled miniprep DNA on a 1% agarose gel.
20. To analyze the size of the deletion inserts, the miniprep DNA samples were digested with Hind III & BamH I. The inserts that gave deletions of 250-bp with a 50-bp overlap were chosen for sequence analysis.

2.2.13 Double stranded DNA sequencing by dideoxy chain termination method
The nested deletion subclones were sequenced using sequence version 2.0 kit (USB) by the dideoxy chain termination method of Sanger et al., (1977).

2.2.13.1 Preparation of double stranded DNA template and sequencing reactions
Double stranded plasmid DNA was prepared by medium scale plasmid isolation protocol described in section 2.2.11.

Sequencing reactions were performed using Sequenase version 2.0 kit according to the manufacturer's instructions using [35S]dATP (BARC, India, Specific activity 600Ci/m mole).

2.2.13.2 Casting sequencing solutions required
Repel silane- 5% dimethyldichlorosilane (Sigma) in hexane. Bind silane was prepared as follows:
Solution A - Silane A174 (Pharmacia) 31.25μl
    Ethanol 6.25 μl
Solution B - Acetic acid 18.75μl
    Water 187.5μl

Solution A and B were mixed and used.

40% acrylamide (19:1) - Prepared and deionised as recommended (Sambrook et al. 1889). 10% APS - Prepared fresh by dissolving 100mg/ml in water.

Routinely 6% sequencing gels were run. The gel composition is:
10X TBE - 15ml
40% acrylamide - 22.5ml
Urea - 63g
10% APS - 600μl
TEMED - 60μl and water added to 150ml.

25ml of gel solution was used for sealing the bottom of the gel apparatus before pouring the gel.

Sequi-Gen Nucleic acid sequencing cell (BioRad) was used for running the gels. Plates used were 38X50Cm dimension. Spacers used were 0.4mm thick. 48 well, 0.4mm thick sharkstooth comb was used to form the wells.

One of the plates bonded to the buffer chamber was treated with repel silane. The outer longer plate was treated with bind silane. The assembled plates were sealed at the sides by clamps and at the bottom with a strip of Whatman 3MM-paper impregnated with catalyzed acrylamide. The gel was cast after degassing the gel solution.
2.2.13.3 Electrophoresis

The gels were prerun at 2500V, 100W for 2-3 hours until the gel temperature stabilized at 50°C. The samples were denatured by boiling at 95°C for 3 min, Spun briefly and mixed. The surface of the gel was flushed with a syringe to remove the undisolved Urea. 2.5μl of each sample was applied to the top of the gel surface with sequencing gel loading tip (Stratagene). Each sample was loaded twice.

2.2.13.4 Preparation of sequencing gel for autoradiography

After the run, the apparatus was disassembled. The plates were separated by a spatula. The glass plate with the gel was fixed in 10% methanol, 10% acetic acid in water for one hr with occasional shaking. The gel plate was then dried in a 60°C oven overnight with the gel side up. The dried gel was exposed to XAR (Kodak) X-ray film by sandwiching the film between the gel and another glass plate support and wrapping it up in a black cloth. The film was developed after 3 days.

2.2.13.5 Reading the sequence

The sequence was read from the bottom of the gel in the second loading and then into the first loading of the same reaction. The sequence was then compiled into one. The overlaps between the successive clones were established. The sequence was compiled using PC gene Pro (GP) software.

2.2.14 DNA sequence analysis and homology search

The sequence of the various subclones was compiled after comparing the sequence of the opposite strands. Differences were sorted out by going back to the autoradiogram. The DNA sequence was compared to the sequences available in the EMBL (Ham and Cameron, 1986) and GENBANK (Biolofsky et al., 1986) databases and homology of the deduced amino acid sequences was searched for all the amino acid sequences in the SWISS-PROT (Bairoch and Boeckmann, 1991) database using Fast A program (Pearson and Lipman, 1988).

2.2.15 Subcloning of deletion product in yeast expression vector (pSZ211)

1 All selected deletion constructs were digested with Hind III and Bgl II. Deleted promoter DNA fragments were purified through agarose gel electrophoresis (AGE) using GeneClean II.
2 pSZ211 vector was digested with Hind III and BamH I. Vector backbone was purified through AGE.
3 100ng of vector and 40ng of deletion products were ligated with T4DNA ligase at 16°C for 16h.
4 Recombinant clones from all deletion time points was selected by screening of miniprep DNA and confirmed by restriction analysis by Hind III and BstY I.
2.2.16 Yeast transformation
Transformation of Yeast cells was done according to Geitz. et.al., 1992

2.2.16.1 Preparation of competent cells
1. A single colony was inoculated into liquid YPAD and grown overnight to 1-2x10^7 cells/ml at 30°C.
2. Culture was diluted with YPAD to 2x10^6 cells/ml.
3. Culture was regrown up to 1x10^7 cells/ml.
4. Cell was harvested by centrifugation at 5000 rpm for 5 min.
5. Medium was discarded and the cell pellet was washed twice with sterile MilliQ water.
6. Cell was resuspended in 1 ml water and transferred to 1.5 ml centrifuge tube.
7. Cells were pelleted and washed with 1 ml TE/Lithium acetate buffer [10mM Tris-HCl (pH 7.5), 1mm EDTA and 100mM Lithium acetate (pH 7.5)] (Solution made freshly)
8. Cells were resuspended as 2x10^9 cells/ml in TE/lithium acetate.
9. The competent cells could be stored at 4°C for 4-5 days without significant loss in competence.

2.2.16.2 Transformation
1. 50 µl of competent cell, 1µg of plasmid DNA and 50µg of sheared salmon sperm DNA (as carrier) was mixed in a microfuge tube.
2. 300µl of sterile 40% PEG (3500) in TE/Lithium acetate was added and mixed thoroughly (PEG and lithium acetate/TE was prepared from 10X stock, just before the addition.
3. Incubated at 30°C with agitation for 45 min.
4. The cells suspension was transferred to 42°C water bath for 15 min.
5. Samples were taken out, allowed to cool at room temperature for 15 min.
6. The cells were pelleted and supernatant was discarded.
7. The cells were resuspended in 0.1ml TE, and plated on SD-His plate.
8. Plates were incubated at 30°C for 2-3 days.
9. Transformants started to appear after 3-5 days.

2.2.17 α-galactosidase assay

2.2.17.1 Filter assay
1. Transformants were patched on SD-his plate and allowed to grow on 30°C for 2 days.
2. Patches were lifted onto nitrocellulose filter
3. Filter was dipped in liquid nitrogen for 2 min.
4. Nitrocellulose filter was transferred on 3MM-Whatman sheet, which was saturated with Z-buffer containing 0.4mg/ml X-gal, kept in petri plate.
5. Plate was incubated at 37°C for 2.6 hr.
2.2.17. 2. β-galactosidase assay from crude extract

β-galactosidase assay was performed according to the method of Rose and Botstein, 1983

A. Extract preparation
1. Three independent colonies from each transformant was inoculated separately in three different flasks in liquid SD-his medium and allowed to grow in incubator shaker at 200rpm at 30°C for 3 days.
2. A 10-ml medium was inoculated to 1% from preculture.
3. Cells were harvested when A660 reached a value of 1.
4. Cells were chilled on ice, and resuspended in 1 ml breaking buffer (100mM Tris-Cl (pH 8.0), 1mM DTT & 20% glycerol).
5. Equal volume of glass beads (0.45-0.5mm) was added and vortexed 10 times at top speed in 30 second bursts with 1 minute incubation on ice between bursts.
6. Liquid extract was withdrawn by placing 100μl pipette tip to the bottom of the tube.
1 Extract was clarified by centrifugation for 15 minutes in microfuge.

B. Enzyme assay
1. 200 μl of extract and 800μl of Z-buffer (Na₂HPO₄.7H₂O, NaH₂PO₄.H₂O KCl MgSO₄.7H₂O and β-mercapto ethanol, pH 7.0) was mixed.
2. Mixture was incubated at 37°C for 5 minutes.
3. Reaction was started by adding 0.2ml ONPG (4mg/ml in Z-buffer)
4. Mixture was incubated at 37°C for 2h.
5. Reaction was terminated by adding 0.5ml 1M Na₂CO₃ stock solution
6. Optical density was taken at A420.
7. Protein concentration in the extract was determined by the dye-binding assay of Bradford (1976).
8. Specific activity of β-galactosidase was determined according to the following formula.

\[
\text{Specific activity} = \frac{\text{OD420} \times 1.7}{0.0045 \times \text{protein} \times \text{extract volume} \times \text{time}}
\]

[OD₄₂₀ is the optical density of the product, O-nitrophenol, at 420 nm. The factor 1.7 corrects for the reaction volume. The factor 0.0045 is optical density of a 1-mole/ml solution of O-nitrophenol. Protein concentration is expressed as mg/ml. Extract volume is the volume assayed in ml. Time is in minutes. Specific activity is expressed as n moles/minute/mg protein.]
2.2.18 Gel Mobility shift assay

2.2.18.1 Preparation of *Collybia* extract

1. *Collybia* culture was grown as mentioned earlier, for 15 days.
2. Culture was induced with 13.5mM oxalic acid for 4h.
3. After 4h., fungal disc was washed with sterile milliQ water and washed once with extraction buffer (50mM HEPES-KOH, pH 7.5, 400mM (NH₄)₂SO₄, 5mM MgCl₂, 1mM EDTA, 10% glycerol, 1mM DTT) and 1mM phenylmethyl sulphonyl fluoride (PMSF)
4. Culture disc was blotted with Whatman no. 3 sheets, to remove adhering buffer.
5. 20gm Disc was grounded in liquid nitrogen by mixer (Remi) to fine powder
6. Powder was transferred to homogenizer vessels and resuspended in 40 ml of breaking buffer
7. Equal volume of glass beads (0.45 to 0.5mm) was added and homogenized by Braun homogenizer in three minute bursts with 1 minute interval.
8. Extract was transferred to SS34 centrifuge tube, and centrifuged at 10,000rpm for 20 minutes at 4°C.
9. The clarified extract was again centrifuged at 100,000 x g (27,500rpm in SW28 rotor).
10. The supernatant was collected and proteins were precipitated by the addition of 100% (NH₄)₂SO₄ (pH 7.5) to a final concentration of 50%.
11. The extracts were incubated for 30 minute at 4°C with shaking and then centrifuged for 20 min at 27000g (GSA rotor at 1400rpm).
12. The protein pellet was resuspended in binding buffer, and labeled as 50% cut off.
13. The supernatant from step XI was made upto 80% (NH₄)₂SO₄ by slowly adding the pre-cooled (NH₄)₂SO₄
14. Extract was incubated for 30 min at 4°C and then centrifuged for 20 min at 27,000g (GSA rotor at 1400rpm).
15. The protein pellet was resuspended in 5 ml of binding buffer. And labeled as 80% cut off.
16. Both the extracts were dialyzed against binding buffer using 8000MW cut off membrane at 4°C for 8-12 h. Buffer was changed at 2 hr interval.
17. Protein concentrations were determined using Coomassie Blue supplied by the (Bio-Rad).
18. Protein sample was stored at -70°C in 100μl aliquots.

2.2.18.2 Target DNA preparation

A. By restriction digestion

The plasmid pBOD was digested with BstE II in NEB buffer at 60°C for 6h, followed by MSc I, Sma I, Ava I, Nsi I, Sac II digestion separately. pBOD was digested with Msc I followed by digestion with Ava I, Sac II separately. All these digestions were performed using NEB enzymes and buffers according to manufacturer's instruction. All these different digests were purified through AGE using Geneclean II Kit (Bio101).
B. PCR amplification

Oligo were designed to amplify the different region of promoter where restriction site is not available, by using PCR-PLAN (PCGENE)

Oligo- OP3, OP4, OP1, OP5, OP2, O03, O04, O12

1. PCR reaction was carried out by Ampli Taq (perkin-Elemer) using PTC-100 (M.J. Research) thermal cycler. Reaction mix was prepared according to suppliers instructions.

2. Cycle condition was as follows:

\[
\begin{align*}
94^\circ C & \quad 2 \text{ min} \\
94^\circ C & \quad 1 \text{ min} \\
54^\circ C & \quad 1 \text{ min} \\
72^\circ C & \quad 1 \text{ min} \\
72^\circ C & \quad 10 \text{ min}
\end{align*}
\]

3. Amplified DNA was checked on 1% agarose gel and purified through AGE using Geneclean II kit (Bio101)

C. Probe preparation

1. Target DNA was labeled by \([\gamma^{32}p]\)dATP using T4 Polynucleotide kinase

\[
\begin{align*}
50 \text{ ng DNA} & \quad 10 \mu l \\
10X \text{ PNK buffer} & \quad 5 \mu l \\
10 \mu Ci/\mu l \text{ Y32PATP} & \quad 3 \mu l \\
100U/\mu l \text{ T4 Polynucleotide Kinase} & \quad 2 \mu l \\
H_2O & \quad 30 \mu l
\end{align*}
\]

All these reagents were mixed properly and incubated at 37°C for 1h.

2. After 1h the reaction was stopped by incubating at 65°C for 20 min.

3. Probe was purified through Sephadex-G50 column as mentioned in Sambrook et al. (1989).

2.2.18.3 Protein - DNA binding reactions

Protein- DNA binding reactions were carried out in a 20\(\mu l\) volume containing 0.5ng (10,000cpm) of probe DNA and binding buffer (20mM Hepes KOH pH 7.6, 40mM KCl, 1mM DTT, 5% glycerol). 40\(\mu\)g extracts were added per reaction supplemented with 20\(\mu\)g dA-dT. Binding reaction were incubated for 10-15min at 4°C. In competition assays, the specific competitor DNA or non-specific competitor pUC19 DNA was added at 0-100 fold molar excess prior to addition of the protein extracts.

2.2.18.4 Resolving the protein - DNA complexes

The protein-DNA complexes were resolved on a 5% polyacrylamide gel (29:1 acrylamide: bisacrylamide), in 1X Tris-glycine EDTA (TGE) buffer. The samples were run on a 1.5mm thick gel in Hoefer apparatus. Gels were run at 150Vfor 4-5hrs until the dye front just ran out.
2.2.18.5 Detection of Protein-DNA complexes by autoradiography
The gels were transferred onto a Whatman 3mm sheet and the upper surface was covered with Saran wrap on the top. The gels were dried under vacuum in a gel drier for 3 hours. The dried gel was exposed to Amersham hyper film with an intensifying screen at -70°C.

2.2.18.6 Phosphorylation analysis
50 µg protein extract was treated with 200 units of calf Intestine alkaline phosphatase (Promega) for 4h. at 37°C in a CIP-buffer, 200 units of phosphatase boiled for 30 minute was used as negative control. Phosphatase treated extract was assayed by gel mobility shift assay using Klenow labeled BM (0.5Kb) probe.

2.2.18.7 Inhibition with Major/Minor groove-binding drugs
The 0.5kb (BM) probe was incubated for 30 min at room temperature with varying concentrations of distamycin A (10µM to 1.4mM), actinomycin D (10µM to 1.4mM) or methyl green (50µM to 1.4mM), drugs that specifically interact with the minor or major groove of the DNA double helix, respectively (Copenhaver et al. 1994; Kim and Norden 1993). The DNA drug mix was then analysed for binding in a gel mobility shift assay.

2.2.19 South western analysis
All operations were carried out at 4°C unless stated otherwise. Partially purified cell extract (100 µg) was fractionated on 8% native PAGE and transferred to nitrocellulose membrane in a buffer containing 25mM Tris and 190 mM glycine at 13 mA for 16 hr. The membrane was incubated in blocking buffer (5% non fat dry milk 25mM Hepes pH 7.6 1mM EDTA and 1mM DTT) for 2 hr and then incubated with binding buffer containing 32P labeled BM probe for 2 hr followed by brief washing and autoradiography.

2.2.20 DNasel Footprinting analysis
1. Target DNA, 0.5kb BM fragment, was labeled by fill in reaction using [γ32P] dATP, dCTP, dGTP, dTTP and Klenow DNA polymerase
   50ng BM DNA 5µl
   10 x Klenow buffer 5µl
   2.5mM dCTP, dGTP & dTTP 6.0µl
   γ32-P dATP 4.0µl
   50/µl Klenow 3.0µl
   Mixed and incubated at 37°C for 1 hr.
2. Reaction mixture was incubated at 65°C for 20 min to inactivate Klenow.
3. Labeled DNA was purified through sephadexG-50 column.
Fig. 2 Northern blot analysis

(A). Total RNA was extracted and electrophoresed on a 1.5% formaldehyde agarose gel. After transfer to a Hybond-N membrane and UV crosslinking, the membrane was hybridized with the random primed -P-radioactively labeled OXDC cDNA probe washed under high stringency condition, and then exposed to X-ray films. Autoradiogram representing the RNA samples prepared from cultures treated with HCl (H), Oxalic acid (O) potassium oxalate (K) and uninduced (U). (B) Induction kinetics, lane 1 showing RNA from uninduced culture and lane 2-10. RNA from oxalic acid treated culture harvested at 30', (lane 2) 1hr (3) 2hr (4), 3hr (5) 4hr (6), 5hr (7), 6hr (8), 7hr (9) and 8hr (10). Equal loading of RNA is shown by ethidium bromide staining (lower panel).
2.2.20.1 Standardization of DNase I concentration

1. 20μl Binding reaction (which contained 50ug protein and 40,000cpm probe) was carried out as mentioned in above, with different amounts of dA-dT (0.5ug, 1.0ug, 2.3.4.ug). Parallely, a binding reaction was also carried out without protein.
2. DNase I was diluted in binding buffer with 1mM MgCl.
3. After binding at 4°C for 20 min, reaction was transferred to room temperature (25°C) for 5 min.
4. 5μl of diluted DNase I containing varied amounts of DNase I was added, mixed thoroughly and incubated at 25°C for 3 min.
5. After that 70μl of DNase I composite stop buffer (7% ethanol, 50μg/ml tRNA and 5% saturated ammonium acetate) was added, mixed and immediately transferred to -70°C for 30 min.
6. Samples were taken out at centrifuged at 13000rpm for 10 min. Supernatant was discarded in radioactive disposable tank.
7. Pellet was washed with 70% ethanol, and dried in vacuum.
8. Pellet was resuspended in 20 μl gel loading dye (98% formamide 0.002% Xylene cyanol).
9. 10 μl sample was loaded in 8% denaturing polyacrylamide urea gel. when xylene cyanol traveled two thirds of the gel, second loading was done, and run was continued until bromophenol blue reached the bottom of the gel.
10. The gel was transferred to Whatmann 3MM paper and dried.
11. The dried gel was exposed to Kodak Biomax MS film and kept in -70°C for 16 hr.

2.3 Result and discussion

2.3.1 Study of Induction

A clone of size 1.3 kb specific to Oxalate Decarboxylase was obtained. sequence analysis of the clone revealed that the 5’ end of the cDNA was missing. The missing portion was later obtained by 5’-RACE-PCR. The complete cDNA was subcloned into pTZ18C and named as pOXDC. Mehta and Datta, [1991] showed using Northern blots, that oxalic acid induces the transcription of oxalate decarboxylase (OXDC). Since, addition of oxalic acid to the culture medium decreases the pH to 3.0, it was of interest to study whether this induction is a function of pH and/or oxalic acid. C. elytica culture was grown for 20 days and induced with 14mM oxalic acid, potassium oxalate and HCl. Total RNA was isolated from induced cultures after 6hr of induction. 20μg of RNA was resolved on 1.5% formaldehyde-agarose gel. One half of the gel was stained with ethidium-bromide for direct comparison of RNA loaded, while the other half was blotted for Northern hybridization. 1.3kb OXDC cDNA insert was used to probe the RNA. RNA samples induced by oxalic acid and HCl gave strong signal where as uninduced and potassium oxalate treated cells showed very faint bands of OXDC (Fig. 2 A). This data
Fig. 3 Mapping of transcriptional start point (TSP) of OXDC by primer extension.
Total RNA from HCl, Potassium oxalate and oxalic acid induced culture, was annealed with labeled oligo O06 and extended with reverse transcriptase. Radio-labeled primer-extended product were then electrophoresed on a denaturing Urea-PAGE gel, along with a dideoxy sequencing ladder prepared using the same primer and pSC24 as a template. Primer extension was carried out at 60°C using rTth (A). Lane H representing HCl-treated, K represents sample from potassium oxalate and O represents sample from Oxalic acid and U represents sample from uninduced cells. (B) and (C) extension carried out using Superscript II reverse transcriptase at 40°C.
shows that there is a basal level of expression of this gene at normal condition, but its expression is induced at low pH of medium. This suggests that the induction of transcription of OXDC is a function of pH and not the substrate as such.

2.3.1.1 Induction Kinetics

In order to know the time required to achieve the steady state level of transcript, the culture was induced by oxalic acid and the hyphal cells were harvested at 0 min, 30min, 1hr, 2hr, 3hr, 4hr, 5hr, 6hr, 7hr and 8hr. following the addition of oxalic acid 20μg of total RNA was resolved on 1.8% formaldehyde-agarose gel followed by Northern hybridization. Fig 2 B shows that mRNA had attained maximum level even at 30 min post induction and remained constant till 8hr.

2.3.2 Determination of transcription start point(TSP)

Fine mapping of RNA structure can be accomplished by a number of techniques. Primer extension, RNase protection assays and S1 nuclease mapping can each be used. Primer extension is used to map 5′ end of the specific RNA and can also be used for quantitating the amount of RNA. In order to map 5′-end or transcription start point of OXDC primer extension was carried out using labeled oligo 006 with 5μg of total RNA. Simultaneously a sequencing reaction was also done and electrophoresed in parallel, with the same primer. Primer extension was performed using superscript II. Fig 3 B shows the primer extension product from different RNA samples. The autoradiogram shows strong signal in HCl and oxalate treated cultures, while uninduced and potassium oxalate treated cultures gave faint signals. The extended product showed multiple bands around ATG, which could be due to the secondary structure mediated hindrance in elongation. The gel was again exposed for longer time. Fig 3 C shows that transcription starts at A located at -11 position with respect to ATG. To further confirm this, primer extension was carried out using T7 polymerase, which catalyzes the reverse transcription at 60°C. At higher temperature, polymerase will not face the hindrance imposed by secondary structure of RNA. Fig 3 A shows the primer extension and sequencing reaction. A single band appeared in HCl and oxalate treated culture. The position of band is similar, as it was obtained by superscript II. Transcription start point is mapped to an adenine residue at -11 position from ATG. The intensity of bands is more in HCl and oxalic acid treated samples, which again potentiates the earlier finding that low pH mediated transcriptional activation of OXDC.

2.3.3 Subcloning of upstream region

To sequence the OXDC upstream region, it was subcloned into pBluescript II KS+. A Bgl II site was engineered at +10 (+1 position is A of ATG), to carry out deletion and the same deletion product can also be used for the translation fusion with β-galactosidase for in vivo assay of promoter activity. Bgl II site was engineered by PCR using oligo O12
Fig. 4  Construction of pBOD and pBODR0
10 kg of supercoiled plasmid was used as a substrate for Exo III deletions. Deletions were allowed to proceed unidirectionally at 31°C and samples withdrawn every 30 seconds. The extent of deletions was monitored on a 1% agarose gel. The size was estimated from the DNA molecular size standards (Lambda DNA digested with Hind III and pUC19 DNA digested with Hind I).

Fig. 5 Unidirectional nested deletions of 2.4kb pBOD (A) and pBODRO (B).
Fig. 6. Nucleotide sequence of the 5′-flanking region of OXDC gene.
The translation initiation codon ATG is shown in bold and italic. The consensus transcription factor binding site, TATA, CAAT, AP-1 SP-1 and C-/EBP are shown in boxes. Oligonucleotide sequence are marked in gray color which have been used in GMSA experiment for probe preparation.
(which flanks from +17 to -7, in respect to ATG, and containing a Bgl II site) and O03 (flanking the region -534 to -515, in respect to ATG). Using these two Oligos PCR was carried out by vent exo- DNA polymerase. Fig 4 shows the amplified product of size 0.55 kb. PCR product was digested with Nsi I and purified, remaining portion of upstream region was taken out by digesting the pSC24 with Nsi I and Hind III. These two fragments were subcloned into pBluescript II KS+ at Hind III and Sma I site. The resulting construct was named as pBOD. To sequence the other strand of the DNA, The same insert was subcloned into reverse orientation. pBOD was digested with BamH I and religated; plasmid for reverse orientation was screened by digesting the recombinants with Kpn I. Digestion of pBOD with Kpn I will give two bands having size of 5.1kb and 0.025kb while pBODRO digestion with Kpn I produced two bands of sizes 2.9 kb and 2.3 kb.

2.3.4 Generation of unidirectional Nested deletions and Sequencing
Plasmids, pBOD and pBODRO were deleted using Exonuclease III at 31°C. Pst I was used as a protection site in pBOD deletion while Sac I was used in pBODRO. EcoR I was used as sensitive site in both the cases. Supercoiled plasmid preparations were used for deletion to prevent the smearing of the DNA, as Exo III can actively digest nicked molecules to near completion. Screening of the deletions gave more than one size of insert at any given time point. It was therefore imperative to check for the presence of the primer-binding site close to the primer site. Sample those overlapped at least by 50-100 bp, were chosen for sequencing. The deletion of pBOD and pBODRO are shown in Fig 5
For sequencing, double stranded plasmid DNA was used as a template. Some templates are problematic for sequencing by Sequenase due to the presence of secondary structure or higher GC, in those cases they were sequenced by vent polymerase.

2.3.5 Sequence analysis
The sequence of both the strands were compiled using GENEPRO sequence analysis package. The nucleotide sequence of the 2280bp region is presented in Fig 6 Analysis of the sequence revealed the presence of TATA and CAAT boxes upstream of the translation initiation sequence. TATA element is present at -38 to -44 bp from the translation start site, consistent with other eukaryotes where it has been reported to be present at -25 to -30 bp from transcription start site (Struhl 1995). The TATA sequence is the binding site for the TATA-binding protein (TBP). TBP-TATA association nucleates the assembly of an approximately 4-MDa-transcription preinitiation complex, a step that can be rate limiting for transcription in vivo (Klein and Struhl 1994). CAAT box is present at -175 to -178 bp from translation start site. The CCAAT-binding factor is heteromeric DNA-binding complex, which is highly conserved from mammalian to yeast
Fig. 7 Construction of pSZOD and deletion series
systems, and these are required for transcriptional activation (IN-Sankim et al. 1996 and McNabb 1997). The promoter region of OXDC also contains a putative SPI binding site at -472 to -477 bp, C/EBP at -432 to 440 and an AP-1 binding site at -274 to -282. (Fig 6).

2.3.6 Construction of vectors containing promoter deletion mutation and their expression:

To identify sequences in the 5′-fanking regions of the OXDC gene important for efficient gene expression, a set of promoter deletion mutants were selected, which, have been used earlier for sequencing. These deletion mutants were translationally fused with E.coli β-galactosidase (LacZ) gene. Since, there is no transformation protocol established for Collybia and it is well known that the transcriptional machinery is highly conserved among yeast, Drosophila and mammals (Guarente, 1988, Guarente, et al 1992 and Yoshinaga, S.K. 1992) e.g. α-amylase gene of Drosophila is repressed by dietary glucose, also showed glucose-mediated repression in yeast. This corroborates the earlier findings on the structural and functional conservation of basal transcription factors among eukaryotes (Hickey D.A et al 1994). Based on the report, S. cerevisiae was chosen for analysis of promoter to identify cis-acting elements.

Plasmid pSZ211, was used for the construction of fusions. pSZ211 contains leu2 gene for prototrophic selection after transformation in leu− strain. Fig.7, is showing outline of construction strategy while, Fig. 8 shows the location and extent of different deletions. Different promoter deletion constructs were used to transform S. cerevisiae His−, leu− (AH22). Transformants were selected for leucine prototrophy. A qualitative analysis of different deletions was performed using X-gal plate assay. Twenty independent transformants were checked from each deletion. Transformants from all deletions showed blue color except the pB18 (-110bp) (Fig.8). Some of the transformants from C23 (-2100bp) and B46 (-500) showed few white colonies while other colonies from same deletion construct gave blue color. This white colony appearance, could be either due to false negative or gene conversion and /or double-crossover event. This type of integration event has been found in other systems as well (Fincham 1989). A quantitative β-gal activity was measured for all deletion mutants. Protein extracts prepared from cells grown in minimal SD medium and SD+14mM oxalic acid. Fig. 9 shows the β-gal activity from different deletions. Values presented are the mean of six independent transformants from each construct. This data shows that the Saccharomyces does not respond to presence of oxalic acid in the medium. Deletion of upstream region form -2227bp to -450bp does not show any significant difference in the
Fig. 8 Qualitative filter assay of β-galactosidase from *S. cerevisiae* transformed with different deletion constructs. Extent of deletion is numbered with respect to ATG.
Fig. 9. Expression of β-galactosidase from different deletion constructs.
Hatched bar are showing the activity from the yeast culture grown in SD and plain bar representing the activity from the oxalic acid induced culture. Values presented here are the mean of six independent transformants.
promoter activity, whereas further deletions showed 34% decrease in promoter activity. Deletion at -160bp shows 60% drop in promoter activity, further deletion abolishes the promoter activity. This suggests that, *Saccharomyces* does not respond to the oxalic acid mediated transcriptional activation, but it is able to initiate a basal level of transcription. A region surrounding the CAAT box is important for initiating the transcription, as it is evident from the data that deletion at few bases upstream region of CAAT box reduces 34% activity. Presence of C/EBP (CAAT enhancer binding protein) binding site upstream of CAAT box suggests that there could be cross-talk between these two factors during transcriptional activation, while CAAT independently can activate the transcription to 60% of the total promoter activity. Further deletion, abolishes the transcription which may suggest that TATA box alone cannot efficiently direct the transcription and it requires the signal from upstream transacting factors. As it has been reported, that yeast CAAT binding factor can regulate the transcription of *AmdS* gene from *Aspergillus* (Bonnefoy et al. 1995). C/EBP interacts with TBP and TFIIB, and activates transcription both in yeast and mammalian cells (Nerlov, and Ziff 1995). C/EBP regulates the transcription by utilizing both the mechanism i.e. activation and repression (Williamson et al. 19998). Deletion construct B18 (-110bp) which contains TATA box does not show any β-gal activity, as it is known that TATA box is sufficient for recruitment of basal transcription machinery, but in most cases polymerase gets paused; further activation requires upstream transcription factors to abolish the pausing, for efficient transcription elongation. Recently it has been shown that CBF (CAAT binding factor) directly interacts with TAF110 through glutamine rich region of its activation domain, (Coustry 1998) and CBF is associated with GCN5 (a histone deacetylase involved in transcriptional activation) (Currie 1998), confirming the role of associated factors in transcription elongation. Similar mechanism might be operating here to carry out basal transcription.

### 2.3.7 Gel Mobility Shift Assay (GMSA)

In order to find out the trans-acting factor(s), which activate(s) the transcription in presence of oxalic acid in the medium as inducer. A number of GMSA were performed using different regions of promoter. Fig.10 shows the various region of promoter used for GMSA, left panel shows that DNA fragments that were obtained by PCR amplification and right panel showing the DNA fragments obtained by restriction digestion. GMSA was carried out using 32P-labeled BM DNA sequence, total cell extract prepared from uninduced, and oxalic acid induced culture. Fig. 11 A, shows a major shift in mobility in induced extract, where as uninduced extract does not show any difference in mobility. This data positively show that there exists a factor, which can bind to this promoter only in of oxalic acid induced protein extract. To test our earlier finding that whether induction of *OXDC* is mediated by lowering the pH of medium total cell extract
Fig. 10. Outline of promoter fragments used for GMSA and DNase I experiment. Left panel showing the fragments isolated by PCR. Right panel shows the fragments isolated by restriction digestion.
Fig. 11 GMSA using labeled BM and total cell extract.
(A) lane 1, Free probe, lane 2 with uninduced extract and lane 3 shows binding with oxalic acid induced extract. (B), showing binding with oxalic acid induced extract. binding was performed with 50-80% cutoff cell extract, and (C), representing binding with 0-50% cut off cell extract. Lane 1 shows free probe and lanes 2-5 shows binding with uninduced, HCl-induced, oxalic acid induced and induced with potassium oxalate respectively.
was prepared from the mycelium induced by potassium oxalate or HCl, or Oxalic acid, and uninduced mycelium. The extract was partially purified by differential ammonium sulphate precipitation. First, the crude extract was precipitated with 50% ammonium sulphate, the pellet obtained was saved as 0-50% cut off and then supernatant was made upto 80% concentration of ammonium sulphate. The pellet obtained in this case was saved as 50-80% cut off. 80% cut off extract shows a strong shift in the induced extracts of oxalic acid and HCl (Fig. 11, B), whereas there is no detectable binding in uninduced and potassium oxalate treated cells. The 50% cutoff extract does not show any detectable shift of band in any of the samples (Fig 11 C). These findings suggest that the factor gets precipitated in 80% ammonium sulphate and is inducible by pH and not by potassium oxalate, consistent with earlier finding.

2.3.7.1 Standardization of Binding in the presence of nonspecific carrier DNA
Different non-specific carrier DNAs were tested for efficient binding, Fig. 12, A, shows the binding performed with sheared Salmon sperm DNA (SSS DNA) and poly dl-dC. The data shows that in the presence of 1μg SSS DNA, the complex starts dissociating, where as concentrations of 1.5 to 2.0 μg SSS DNA completely dissociates the complex. However, poly dl-dC is not suitable for binding at all, because even in the 0.5μg range the complex is not formed properly and at 1.0μg concentration, the complex formation was completely abolished. The binding was not affected in the presence of pUC19 used as a non-specific competitor (Fig. 12 B). Poly dA-dT shows a long range of binding, the complex formation was not disturbed upto 2.5μg concentration, and further higher concentrations show that the complex starts to loose (Fig.12C).The complex dissociation in the presence polydl.dC and SSS DNA can be explained by the fact that the target site may be a GC rich sequence (Later DNasel protection experiment revealed that target site TCGGCGACCCCGC, contains a GC rich sequence) . Two different pH values were tested for complex formation, and it showed that complex formation is not affected at pH 7.0 and pH 7.7. (Fig 12C).

2.3.7.2 A saturation kinetics
Binding was performed using 1ng of probe and varying amounts of protein. The binding was performed with uninduced extract and induced (with oxalate) extract. Fig.13 A&B, shows that the binding site is saturated at 40μg protein concentration. Similarly, binding was performed with the extract induced by HCl, which also showed similar result (Fig.13 C).

2.3.7.3 Competition experiment
To confirm that the complex formation represents a specific DNA-protein interaction, competition experiments were carried out. Addition of excess unlabeled homologous DNA fragment (probe fragment) to the binding reaction competed for complex formation (Fig. 14 A, lane 3,4.), lane 3, with 50 fold excess and lane 4 with 100 fold excess. Competition assay with 1000 fold excess of pUC19 did not affect the complex (Fig. 14 A, lane1). These result suggest that binding is specific.
Fig. 12. GMSA with different carrier DNAs.
(A), binding with sheared Salmon Sperm DNA and Poly dl-dC. (B), binding with pUC 19. (C), binding with poly dA-dT and binding at different pH.
Fig. 13 Binding reaction with increasing concentrations of protein extract. Binding was performed using 1 ng labeled probe and 2 μg dA-dT. (A), binding with uninduced extract; (B) binding with oxalic acid induced extract; (C), binding with uninduced and HCl-induced extract.
Fig. 14 Competition assay with specific probe.
Binding reaction was performed using 40μg of crude extract and 2μg dA-dT and binding was challenged with different fragment of cold promoter DNA. (A), lane 1 without competitor DNA; lane 2, with 1μg of pUC19; lane 3, 50 fold BM; lane 4, 100μg cold BM; lane 5, 100 ng cold AM and lane 6, 100ng cold BA; lane 7, 100ng cold SN. (B), lanes 1-8 contain 100ng of cold SM, BN, OP51, OP53, OP33, OP32, OP41 and BS.
Fig. 15 GMSA, Binding with different labeled promoter fragment (A-G). Names of the fragments are shown on the top of each column. F, free probe, U, represents the binding performed with uninduced extract and O, showing the binding reaction with oxalic acid induced extract. E, Lanes 1-4 showing free probe, binding carried out with uninduced extract, with oxalic acid induced extract and binding with oxalic acid induced extract and 50ng cold OP31 respectively. G, Free probe (F), Induced extract (lane 1), binding challenged with homologous DNA (lane 2).
2.3.7.4 GMSA with different labelled promoter fragments

In order to delineate promoter which is sufficient for binding, various DNA fragments which constitute the promoter were used for competition experiment. Complex formation is unaffected with all the promoter fragments except OP51 (Fig 15 B, Lane 3). OP51 covers the entire region of BM DNA fragment.

To confirm, furthermore, binding was performed with $^{32}$P-labeled DNA fragment, which have been used for competition above. There is no detectable complex formation in BA, AM, SN, BN, SM and BS probes (Fig. 15 B, C, D) and OP53, OP32, OP41 and OP42 probe fragments (Fig. 15 F). Binding was observed in A$_{38}$ DNA fragment, which spans from ATG to -1100 (Fig. 15 A). OP51 shows strong binding (Fig. 15 G lane1), the binding was specific as it can be competed out with 10 fold molar excess of cold OP51 (lane 2).

The DNA fragment OP31 also showed the specific binding as it could also be competed out by homologous DNA fragment (FIG 15 E). These data suggest that, the smaller DNA fragments are incapable of complex formation which, could either be due to the importance of DNA topology and secondary structure in protein DNA interactions or the fragment tested is devoid of their companion cis-element. It is well documented in literature that protein-protein interactions are important in complex formation and stabilization.

2.3.7.5 Binding and ionic interaction

GMSA were carried out to determine the interactions involved in factor binding

1. When binding reaction was carried out using the BM probe in the presence of increasing amount of NaCl, complex formation gradually decreased as a function of salt concentration (Fig 16 B). Complex formation can take place even upto 2.0 M concentration of NaCl. This suggested that ionic interactions might be only marginally involved in complex formation.

2. When binding reaction was performed in the presence of different concentrations of EDTA, the complex remained unaffected and was stable even at concentration of 100mM EDTA (Fig. 16A), suggesting that divalent cations are not required in the complex formation.

3. It is well documented in literature that transcription factors are activated by phosphorylation or dephosphorylation events as a result of effector mediated signal transduction pathway. To find out whether this factor is regulated through phosphorylation event, GMSA was carried out using phosphatase treated, and boiled phosphatase treated extract as a control. (Fig.16C). Lane 1 showing the normal binding without treatment, Lane 2 contains extract with boiled phosphatase. Lane 3 and 4 contain binding with phosphatase treated extract. The complex in lane 3 was challenged in the presence of 50-fold excess of specific competitor. These results suggested that complex formation is not affected by phosphatase treatment.

4. GMSA was carried out to determine whether the factor binding to DNA element interacts through the major or the minor groove of DNA double helix. Binding
Fig. 16  GMSA showing effect of salt, EDTA and Phostpahte on Binding

Binding reactions were carried out with BM probe and oxalic acid induced extract (A), Binding carried out at NaCl concentrations ranging from 0.1 to 2.0M., (B), Binding reactions were carried out with varying concentration (1-100mM) of EDTA. (C), Phosphatase treatment, Lane 1 showing the binding without any treatment of extract; Lane 2, binding with boiled phosphatase treated extract; Lane 3, binding with phosphatase treated extract and challenged in the presence of cold BM; Lane 4, Binding with phosphatase treated extract. FP, free probe.
Fig. 17 GMSA in presence of DNA binding drugs.
Binding reaction was carried out with BM probe and oxalic acid. Binding was performed with varying concentrations (0.01 to 1.4 mM) of methyl green (A); Actinomycin D (B); Distamycin A (C)
reactions were carried out in the presence of Actinomycin D and Distamycin A which specifically bind to the minor groove, and methylgreen that interacts with the major groove. Incubation of radiolabelled BM fragment alone with Actinomycin D decreases its mobility, while incubation with methylgreen and Distamycin A did not affect the mobility of the free probe. The DNA-protein complex formation was not affected when probe was incubated in Actinomycin D or Distamycin A (Fig. 17, B and C). The complex formation was inhibited by the methyl green in dose dependent manner. (Fig 17A). These results clearly demonstrate that protein factor interacts through major groove of DNA.

2.3.8 South-Western analysis

Southwestern analysis of the total cell extract from uninduced and induced mycelium was performed to determine the molecular mass of the factor. Cell extracts were fractionated on 10%SDS-PAGE and 8% native PAGE, blotted on nitrocellulose membrane and probed with labeled BM DNA fragment. SDS-PAGE did not show any signal while native-PAGE showed a single band of Mr 280kDa in induced extract while uninduced did not give any band (Fig. 18). This result points the specificity of DNA-binding protein in southwestern analysis. Absence of a signal on SDS-PAGE could either be due to irreversible denaturation of protein or other associated factor, which is incapable of binding to the target DNA element alone. As it is known that most of the transcription factors are tightly associated with the co-activators (Gcn5, Srb5) and mediators that are necessary for binding to target DNA.

2.3.9 Foot printing

In order to find out the sequence recognized by the factor for binding, DNase I footprinting was carried out. Different concentrations of DNase I was used to standardize the optimal concentration of DNase I for ladder formation. 0.06 unit, 0.08, 0.1, 0.2, 0.4, 0.6 0.8 and 1.0 units of DNase I were used with 1μg and 2μg poly dA-dT with or without cell extract. Fig 19 A&B, shows the ladder obtained by DNase I. Ladder was resolved by loading the sample twice to localize any upstream DNA binding protein. Left panel in Fig. 19A,B shows the ladder without extract, lane 1 to 8 representing increasing concentration of DNase I. 1μg of poly dA-dT was used in reaction with free probe, while 1 and 2μg dA-dT was used with protein extract; in lanes 1-8 and 9-18 respectively. Ladder with protein extract gave two windows on comparison with free probe. Uniform ladder was obtained with all the concentrations tried.

In order to obtain the sequence, DNase I protection assay was performed using BM probe. Its coding strand was labelled with α-32P using Klenow DNA polymerase. Sequencing was performed according to the Maxam-Gilbert chemical cleavage method. BM probe gave a good protection, but sequencing did not work, it gave bands in all the
Fig 18. South western analysis.
40μg and 80μg of crude extract of uninduced and induced cultures were electrophoresed on 8% Native PAGE. Protein was blotted onto nitrocellulose. The blot was probed with BM probe.
Fig. 19 DNase I treatment
The free probe and binding complex was digested with varying concentration of DNase I, using dA-dT as specific DNA. Ladder was resolved on denaturing urea/PAGE. Filled triangular bar representing the increasing concentrations of DNase I. Panel A showing the first load and panel B showing the second load. DNase I digestion of induced extract, contain with 1µg of poly dA-dT (lane 1-8) and 2µg poly dA-dT (lane 9-16).
Fig. 20 DNase I protection assay was perform with BM probe and OP51 probe

(A), DNase I protection assay of BM probe in the presence of 40 and 60μg of induced extract and without extract. (B), DNase I protection assay of OP51 in the presence of 40 and 60μg of induced and without extract. G, A, T, C represents the sequencing ladder which was carried out by labeled OP1 primer using pBOD as template. (C), An enlarged representation of footprint area. The protected sequence is shown in uppercase letters while flanking sequence is shown in lowercase letters.
**Fig. 21. DNase I protection assay using labeled BM and BA probe.**

First 3 lanes at left side represent the ladder from BA probe. Last two lanes represent the DNase I digestion of BM probe carried out in the presence of 40 and 60μg of extract.
lanes at same position. This uniform ladder could be due to the nicking of probe during its preparation.

In order to get sequence, the probe used in DNase I protection reaction was prepared by PCR using labeled OP1 primer, and unlabelled OP5 primer. Sequencing reaction was performed with the same labeled OP1. Footprinting of BM probe with coding strand is presented in (Fig.20 A) while footprints obtained by OP1 probe with non-coding strand is presented in (Fig.20.B). Sequence with the same primer is presented at right. footprint obtained from coding and non-coding strand shows a region of 13 nucleotides (STCGGCFACCACGC). The DNase I hypersensitive site is shown by asterix, which is characteristics of DNA binding protein induced hypersensitivity.

In order to confirm that the factor can protect the site by DNase I in BA probe which also contains the target site but is shorter in size; DNase I protection assay was performed. Left side of the column (Fig.21) shows the reaction with BA probe. It did not give any protection, which is consistent with the earlier data where BA probe failed to show any complex formation. These data show that factor protects a 13bp site only in BM probe. Database search of the protected sequence did not match with any known transcription factor or DNA binding factor sequence. However, the sequence obtained was found to be present in the promoter of chicken H2B (Histone 2B) and IGFIIB (Insulin like growth factor II B).

2.3.10 Sequence analysis and expression of C-5 sterol desaturase

Upstream OXDC sequence analysis revealed the presence of C-5 Δ sterol desaturase. Ergosterol is predominant sterol of fungi is postulated to have many cellular functions apart from its role in membrane fluidity, porosity and stability (Taguchi et.al. 1994), ergosterol is essential for growth at low temperature, and it is important as permeability barrier against various chemical stresses (Hemmi et al. (1995) and finally like cholessterols, ergosterol exerts regulatory effect on gene transcription in S. cerevisiae (Smith et al.1996). Studies with sterol auxotrophs show that, even in the presence of sterols which can fulfill the bulk membrane requirements, a small concentration of ergosterol is absolutely necessary for growth. The Δ 5-double bond of the ergosterol has been implicated in regulatory function (Goldstein and Frye 1996). Cloning and sequence analysis of C-5 Δ sterol desaturase have been reported from yeast (Arthington 1991), human (Matsushima et al. 1996) Arabidopsis (Gachotte et al 1996). Sequences from Candida and S. pombe have also been submitted to GENBANK. All of them show very good homology to each other. BLAST analysis revealed the presence of C-5 sterol desaturase upstream of OXDC promoter. Fig 22 shows the complete sequence of C-5, sterol desaturase, which spans a region upto 1.8 kb. The ORF starts from position 759 to 1763. 1st intron is 48 bp long and spans the region from 1189 to 1237. Second
GAATTCCAAATGGAGTGGCACCTACCTGTGGCGCCGAGAATCAGTACGTTGGTGGGAGAC
ATTGGACTGACAGATGACTTGCATTCATTCTAATGTGGTCTTTATATATACCAAGAAACG
TCAGTGGATGATTGATTCAAATCGGTGATGATAGACAGTGCCGGAAAGGTACGTGCCATA
GCATGGCAAAAGAAGTTCAGCCGATTGAATTGACTGGAGCGCACTGGTTATTCAGCTGAT.
CAGGTACGAGTACGGCAATTCCACCGCGCGTACTACGCCACATATCATTGTGGTACAACA.
GTGTGGGCGTCAAAATGAACGGAGTGTATTGACGGCCAAGTCCCGGAGTATTCGGGATAT
TCGGACATTGATAATTGATTATGGGATGCGTAGCGCAAGATGCGTCAACGGGAGCGTCGT
AACCCCGGATTGATCATGGATGTGGCGTAGAAGACTGTCATTCGACCTTTCAAATCATTA.
TTTGCTAACGAGGAATGTCGGCCTCCAGAGCTTTTAATAATGAAAAAACTGGTAGATACA.
AATACTCTCACCTCAACACATCTGCTGTGACACCATATAAAAATGTGAGCTCTAGAGT
CGAATGGTTCACGGAAGGCCTGGCGAAAGAGCCAGACTGGGCCGGTCCGGGTTCCATGTT
CGACCACGTGGTGCGAAGTGGTGCGACACAAATTTTTIIIIIIIIII.GTAAATCGGACTTGT
CTTTTGGTTTGCAACCACCACCACCGCGACGAGCAGCCATGGACGTCGTTCTCAACATCG
MDVVLNIA
rXGACGACTACGTTCTCGACAAAGTCTGGTCGTACATAGTGCCrTTGACGACGAACGCGA.
D D
Y V
L D K
V W 0 Y
I
V P
L T T N A
T
CGCACTGGGAGCCAGCCAGCAATACTAC:CTCACCGTGTCTGCATGGCCTCGCGACTACA
H W E P A S N T T L T V S A W F R D Y I
TTCCGCGCCAGCTGGTCTCTCTCTGCACAATAACGCTCATCGGCATCCATATTCTCTATT
P R Q L V S L C T I T L I G I H I L Y F
TTGCTTYGCAATACCACTCCTACAACAAAAGATATTTAACCACACATGATGCGCCATCGAC
AF A Y A S Y K W I F N H D M M R H F R
GCTTTCTAAAGAACAAGGCGCTTTGAGATTATGCAGCGCTCAAGGCGATTCGCGGGGA
FL KN Q V R L E I M T S L K A F P G M
TGATCTCTTACATTAACTGTTGGCGGGAAGCTAGGGCTACACAGGACTGTAUOS
ML L T L P W F Q A E V M G Y S R L Y E
AGGGATGGCACAFCAGGCTACTACCTACCTGGTCTGAGGCTGACCTATTGTGACTGG
GL D T Y G T Y L V L S V P L
tgttatggcaatgctttcttaAGCTTTTCCCTCCTTCTTACAGACTACC
FL LE T D Y L
TGGCTCTGCGTGGCGACAGAAACTACAGGCTCAGGGTCTTTTACAGGCTGGACAGG
V Y V W N R I L H V E V E Y K A L H K F
CTCATACAAATGGATACGATTGTTATGGTTTCTCTACGATTTTTGCTATTACGAC
H K K W I I
cctcgattACGTCTCTACAGACCGATACGTTTCTTACTACGGGCTACTGCTCTTCTGCTCT
PT F A S H A F H P V T L S S
AATCCCATCCCATCATTTTTCTCTAATTCCCTCTCATGCGCATCTCTATCAAATTTTTAAGCGCA
IM F I S S R L H I P S P S H S L S Y
TTTTTTTGGGGGGTGACCTTTTGGAACTCTTGGACATGCTGCGAGATGACCG
S E R G Q L L D H P Y S R L R H D H R
GGCACCACCTCGAAAACCTCATCATGAGGCACGGCGAACATACCTACACCAGACATCTATT
A P A R N F H Q W F R A P F Y F T P H L F
Fig. 22. Nucleotide sequence of C-5 sterol desaturase.
Intronic sequence are presented in bold letters. 5’ and 3’ splice sequence are shown in upper case, bold italic and branch point sequence are boxed. The putative TATA like element is also boxed. Amino acid sequence is presented below the nucleotide sequence. A putative polyadenylation sequence is boxed.
Fig. 23. Sequence alignment of Collybia, S. pombe, Saccharomyces and Candida C-5 sterol desaturase by ClustalW. Star represents the identical aminoacid; double dot, similar amino acid and single dot, represents the conserved amino acid.
Fig 24. Northern blot analysis
Total RNA was electrophoresed on 1.5% formaldehyde agarose gel and probed with first exon (A) and third exon sequence (B) of Sterol-desaturase. M represents the labeled 1 k ladder. U, RNA sample without any treatment; O, RNA sample from oxalic acid treated culture; K, RNA sample from potassium oxalate treated culture.
intron is 53bp long and starts at 1338. The 5' and 3' splice junction is highly conserved when compared to other eukaryotic splice site.

Translation of the sequence in all three reading frames revealed the presence of two major ORF. First ORF appeared in third reading frame while 2nd ORF appeared in the 2nd reading frame. Both the ORFs show a 30% homology with Candida, S. pombe and Saccharomyces C-5 sterol desaturase. Amino acid sequence of the first ORF is homologous at the N-terminus while second ORF matched at the C-terminal region of sterol desaturase. Analysis of sequence between these two ORFs revealed the presence of two small introns separated by a short exon. Both the introns comply reasonable well with the fungal intron-exon boundary and lariat consensus sequence (Csank et al 1990). The lariat sequences are present 7bp and 12bp upstream of 3'-splice site in 1st and 2nd intron respectively. The promoter region did not contain the classical TATA and CAAT box, it is now well documented that most of the houskeeping genes contains TATA less promoters. However, there is putative TATA like element which have been reported from viral promoter is present at -51 to -61. Upstream region contains several GATA factor binding site. The deduced amino acid sequence was aligned with C-5 sterol desaturase sequence from other sources using ClustalW (Fig.23). It shows a strong homology of about 30-35% among each other. The sequence showed 32% homology with S. pombe and 31% homology with Candida desaturase. Three histidine rich motifs (HX3H, HX2H and HX3H) were also present in the ORF. These histidine rich motifs are characteristics of many plant membrane-bound fatty acid desaturases (Gachotte et al 1996). Since it is involved in the stabilization of plasma membrane structure and function, and the gene is present in close proximity with oxalate decarboxylase which is regulated by external pH; it was of interest to know whether C-5 sterol desaturase expression is also modulated by external pH. Northern analysis was performed using total RNA isolated from uninduced, oxalic acid and potassium oxalate induced cultures. Fig.24 shows the Northern blot of C-5 sterol desaturase. Fig 24 A, shows the blot which was probed by 1st exonic sequence and Fig. 24 B, represents the blot probed by 3rd exonic sequence. The result show that the transcripts are equally present in all the tested conditions, which suggest that the C-5 sterol desaturase expression is constitutive and not influenced by external pH.