LIGNIN CONTENT AND ITS RELATIONSHIP WITH PEROXIDASE ACTIVITY IN EUCALYPTUS STEM

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Abstract

Eucalyptus has increasingly been cultivated for its various economic as well as ecological benefits. The quality of Eucalyptus wood largely depends on its lignin content. The lignin biosynthesis on the other hand is associated with peroxidase (PX) activity. In the present investigation an attempt has been made to find out the relationship between PX activity and lignin content. The study was done for one year from May-98 to April-99. Minimum PX activity was observed in December which gradually increased reaching its maximum in October with marginal variation in July. Lignin content followed similar trend indicating close association of peroxidase activity with lignin biosynthesis.

Introduction

In recent years Eucalyptus has been increasingly used in the afforestation programme not only for it’s economic benefits, but also for some ecological reasons like quick biomass production, soil conservation etc. Eucalyptus wood is used for making quality paper pulp whereas better lignification is needed for making good timber (Kling8).

In plant cells peroxidases have been detected in vacuoles, tonoplast, plasma-lemma as well as inside the cell walls. Cell wall peroxidases described as catalytic agents of the last step of lignin biosynthesis i.e. the polymerisation of cinnamic alcohols (Pang et al16). \( \text{H}_2\text{O}_2 \) has been postulated as oxidant reagent and its formation is mediated by certain peroxidases (Imberty et al.7). Significant peroxidase activity in bean callus was reported by Abates and Biles1. More than sufficient peroxidase activity in bean callus was reported to account for the amounts of lignin induced, but no evidence was obtained that peroxidase was the particular oxidising enzyme used for the final stage of lignin formation (Hadden and Northcote5). On the contrary Harkin and Obst.6 reported that apparently peroxidase is the only enzyme that polymerises p-coumaryl alcohols to lignin in trees.

In the present investigation an attempt has been made to understand the involvement of peroxidase in the formation of lignin, a determining parameter of the quality of wood in Eucalyptus.
### Table 1

Seasonal changes of peroxidase activity and lignin content in Eucalyptus stem.

<table>
<thead>
<tr>
<th>MEAN</th>
<th>P5</th>
<th>P4</th>
<th>P3</th>
<th>P2</th>
<th>P1</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUG</td>
<td>900.0</td>
<td>680.0</td>
<td>950.0</td>
<td>314.0</td>
<td>101.0</td>
</tr>
<tr>
<td>OCT</td>
<td>900.0</td>
<td>680.0</td>
<td>950.0</td>
<td>314.0</td>
<td>101.0</td>
</tr>
<tr>
<td>DEC</td>
<td>900.0</td>
<td>680.0</td>
<td>950.0</td>
<td>314.0</td>
<td>101.0</td>
</tr>
<tr>
<td>FEB</td>
<td>900.0</td>
<td>680.0</td>
<td>950.0</td>
<td>314.0</td>
<td>101.0</td>
</tr>
<tr>
<td>MAR</td>
<td>900.0</td>
<td>680.0</td>
<td>950.0</td>
<td>314.0</td>
<td>101.0</td>
</tr>
<tr>
<td>APR</td>
<td>900.0</td>
<td>680.0</td>
<td>950.0</td>
<td>314.0</td>
<td>101.0</td>
</tr>
</tbody>
</table>

**LIGNIN**

**SPECIFIC ACTIVITY OF PEROXIDASE**

**(mg protein)**

**SAMPLE**

**PLANT**

Note: All data are experimentally determined.
Materials and Methods

Five Candidate Plus Trees (CPTs) of *Eucalyptus* sp. were selected from an existing 15 years old plantation in the district of Purulia based on morphological features such as clear, without twisting bole, diameter at chest height, basal diameter etc. A portion of a twig was used as plant material. The twigs were collected from the 1st branch of each of the selected trees at regular interval extended from May 1998 to April 1999. Collected samples were kept in polybags and were preserved in freezing temperature for subsequent use.

Extraction and assay of enzymes

0.5g of plant material was washed with distilled water and blotted properly and then homogenized with 0.1M sodium phosphate buffer, pH6.5 containing 0.2% polyvinylpyrrolidone and 0.4% Triton-X under ice cold condition. The extracted material was centrifuged at 12,000g for 15 minutes at 6°C. The supernatant was used as enzyme source.

Assay of peroxidase and the estimation of protein was done following the methods of Chen and Asada and Lowry *et al.* respectively.

Estimation of lignin content

Lignin was extracted as lignin thioglycolic acid (LTGA) following the method of Bruce and West with certain modifications. Ethyl alcohol was used for the preparation of alcohol insoluble residue (AIR) and afterwards AIR with 2(N) HCl and thioglycolic acid was put in an autoclave at 15 lb. pressure for 30 minutes instead of boiling in waterbath for 4 hours.

Results and Discussion

The present investigation was done to understand the relationship if any, between peroxidase and lignin formation in *Eucalyptus* plant. Both the parameters i.e. peroxidase activity and corresponding lignin content was measured at regular interval from May '98 to April '99. It is evident from Table 1 that the specific activity of peroxidase was minimum in December in all the five plants. The activity gradually increased thereafter up to May. It showed marginal decrease in July and then again increased showing maximum value in October. The change in lignin content followed more or less the same pattern. The only deviation from the trend was observed in August, unlike peroxidase activity which showed such deviation in July.

Five selected CPTs were used in this study. At any point of time when the observations were made the individual plants showed variation amongst themselves with regard to both the parameters. Yet the mean performance of all these plants with regard to these characters depicted identical trend (Fig. 1 & 2) as was observed in any of the CPTs studied.

The data clearly exhibits close association between lignin content and peroxidase activity. The increase in peroxidase activity from December onwards followed concomitant increase in lignin content also. Polle *et al.* while working in Norway spruce observed the involvement of peroxidase in the production of biopolymers like lignin.

The increase in peroxidase activity from December onwards was possibly due to the rise in the environmental temperature. The
Fig. 1: Mean performance of specific activity of peroxidase in stem of five Eucalyptus trees during May to April.

Fig. 2: Mean performance of lignin content in five Eucalyptus trees during May to April.

deviation that was observed in July for peroxidase activity was likely to be caused by sudden drop in temperature due to rain. Similar trend of peroxidase activity was reported by Das et al. in Eucalyptus leaves. The activity of both the cytoplasmic and cell wall peroxidase in poplar was found by Imberty et al. to be maximum in growing season and minimum in the dormant season. This observations also supports the findings of the present study.

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References

Biochemical Parameters of Lignification in Eucalyptus: Variation, Patterns and Potential Applications

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Abstract

Biochemical parameters of lignification including Peroxidase (Px) activity and phenol content were examined in Eucalyptus plants from different regions of West Bengal. Relative lignin content of stems was found to have a positive correlation with Px and coniferyl alcohol oxidase activity. Lignification related parameters were found to exhibit a spatial trend being lowest in the apical branches and highest in basal branches. Px activity in mature trees gradually increased to a peak from February to October and then declined in December. Eucalyptus Px was found as one major isozyme with a possible minor isozyme. 66 fold purification of Px was obtained and showed differential activity with the substrates NADH, Coniferyl alcohol and Syringaldazine.

Keywords: Peroxidase; Phenol; Lignin; Purification; PAGE

1. Introduction

Eucalyptus is extensively cultivated in India under different agro-climatic conditions for soil reclamation, fuel, timber, paper pulp, essential oils etc. Though economic returns from Eucalyptus is very high, no concerted effort has been made for rapid screening of clones potentially capable of giving high returns in terms of paper quality, good quality timber, fast growth etc. In particular, enzyme characteristics, which might provide for rapid screening techniques for identifying suitable clones, would be useful. The lignin content of Eucalyptus largely determines its timber and pulp quality. The enzymic pathway of lignification needs investigation as a route for rapid screening of clones.

Plant peroxidases (Px) occur in various isozyme forms. Some of these isozymes mediate lignification while others form a response element to different stress conditions. Various workers have identified the Px isoforms responsible for
lignification and cloned plants that over-express Px with higher lignin content have also been raised (Lagrimini et al. 1987). The activity of Px with different synthetic (e.g., o-dianisidine, syringaldazine) or natural (e.g., coniferyl alcohol) substrates has been utilized for detecting isoforms responsible for lignification or generalized activity.

Based on this background the following approach was adopted.

- Selecting Eucalyptus trees from different regions of West Bengal and analyzing leaves and stems for Px, relative lignin content, phenols and coniferyl alcohol oxidase (COD) with a view to correlating selected trees with these biochemical parameters.
- Screening for isozyme (Px) patterns in CPTs with a view to identifying differences if any.
- Attempting to correlate Px isozyme patterns in trees and their progenies with a view to understanding the efficacy of Px as a reliable biochemical parameter for screening clones.
- Attempting clonal propagation from the screened trees with a view to raising cloned plants with the desired biochemical characteristics as given above.

2. Method

2.1. Selection of trees

In consultation with forest department, Govt. of West Bengal and ICFRE, preliminary information was gathered about the existing Eucalyptus plantation in different parts of West Bengal. After a preliminary survey, four locations: Purulia, Joypur, Arabari and Sambdiha were selected. In these locations, superior trees were identified on the basis of morphological characters namely tree height, girth, diameter at breast height and the age of the plants. The selected trees were labeled.

2.2. Mode of sampling

Plant samples were collected at regular intervals from the selected trees in each location. Leaves were collected from the apical and basal part of a secondary branch along with the portion of stem, from each of the selected trees in a location. The plant samples thus collected were preserved at freezing temperature and subsequently used for biochemical estimation.
2.3. Assay of biochemical parameters

Homogenates were prepared and assays carried out according to published procedures as follows: Px was assayed according to Chen and Asada (1989), coniferyl alcohol oxidase was assayed according to Pedreno et al (1989), relative lignin content was estimated by the method of Bruce and West (1989), phenol content was estimated by the method of Mallick and Sing (1980), Syringaldazine oxidase was assayed according to Imberty et al (1985), and NADH oxidase was estimated by the method of Ishida et al (1987).

2.4. Polyacrylamide gel electrophoresis and isozyme detection in different systems

Native gel electrophoresis was carried out in leaf homogenates (prepared as detailed in Section 2.6) from CPTs and seedlings by the method of Rodbard et al (1974). Staining for Px activity was according to the method of Chen and Asada (1989).

2.5. Mode of sampling for gradation studies

Three suitable Eucalyptus plants bearing distinctive numbers 14846(20), 54(2) and 22(1) were selected, from a newly raised Eucalyptus stand at the Calcutta University Agricultural Farm, Baruipur. Five suitable branches from base to apex of each plant were tagged for collection of plant samples. Sampling was done, at one months interval, for six months, extending from January 99 to June 99. Collection and analysis of samples was as detailed in 2.2 and 2.3.

2.6. Purification and characterization of peroxidase from Eucalyptus leaves

Plant material: Basal leaves from secondary branches were collected from a selected tree (P5, Purulia) and processed as below.

(All steps shown below were carried out at 4°C.)

Step 1. Crude - extract preparation: 100 g of thoroughly washed and blotted leaf material was cut and homogenized using 100 mM Na - phosphate buffer (pH-6.5) containing, 0.2% polyvinyl pyrrolidone and 0.4% Triton X-100. The homogenate was then centrifuged at 12000 rpm for 15 minutes and clear supernatants were used as the enzyme source.

Step 2. Ammonium sulfate fractionation: Powdered ammonium sulfate was slowly added to the crude extract with continuous stirring to give 80% saturation. The mix was centrifuged at 15000rpm, for 15 minutes. The precipitate was collected and
resuspended in 100mM phosphate buffer (pH 6.5). The solution was then dialyzed versus 10mM phosphate buffer (pH 6.5) overnight.

**Step-3 Sephadex G-50 Chromatography:** The dialyzed solution was loaded on to a Sephadex G-50 column (1.5 cm diameter, 60 cm long) previously equilibrated with 50...
mM phosphate buffer (pH 6.5). The column was washed with the same buffer. Active fractions with high peroxidase activity were combined and concentrated by lyophilization.

**Step-4 Ion exchange chromatography on DEAE column**: The lyophilized solution from Sephadex G-50 was loaded on to a DEAE column (2.5 cm diameter 20cm long) previously equilibrated with 25mM phosphate buffer (pH 7.2). The column was washed with the same buffer, and eluted with a linear gradient of 200-800mM NaCl in 25mM phosphate buffer (pH 7.2). Active fractions were combined and concentrated by lyophilization.

**2.7. Tissue culture of selected CPTs**

Juvenile shoots and young shoots of selected trees were collected from Purulia and Midnapur regions. Explants were cultured according to the method described in ICFRE bulletin (Published by the Director, Forest Research Institute).

**3. Results**

**3.1. Seasonal variation**

Px activity in both apical and basal leaves has been studied periodically from February to December. Significant variation was observed with regard to spatial distribution and seasonal variation (Fig 1). Basal leaves showed higher activities over the apical leaves at all the time points studied but the change in the apical and basal leaves followed the same pattern. The basal leaves being fully extended and matured showed optimum Px activity. From February onwards peroxidase activity gradually increased reaching its peak in October and then abruptly declined in December. The rate of increase was not uniform throughout the period studied. From February to May there was steady increase, thereafter till July, peroxidase activity remained more or less similar. Thereafter it increased at a faster rate showing maximum value in October.

**Table 1. Purification of Peroxidase from Eucalyptus leaves.**

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg/ml)</th>
<th>Specific gravity (Units/mg protein)</th>
<th>Total unit</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>0.48</td>
<td>1.852</td>
<td>88,896</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>1.36</td>
<td>5.856</td>
<td>31,856</td>
<td>3.181</td>
</tr>
<tr>
<td>Sephadex G-50</td>
<td>0.4273</td>
<td>11.804</td>
<td>12,609</td>
<td>6.373</td>
</tr>
<tr>
<td>DEAE</td>
<td>0.05</td>
<td>123.185</td>
<td>9,238</td>
<td>66.51</td>
</tr>
</tbody>
</table>

VIII
Fig-2. a-d. Gradation of different parameters related to lignification in *Eucalyptus*:

- **2.a. Activity of peroxidase**
- **2.b. Phenol content**
- **2.c. Lignin Content**
- **2.d. Lignin content**

*Note the different parameters and their graphical representation.*
3.2. Corelation of peroxidase with lignin and its related parameters

The trend for Px was the same in both leaf and stem of any individual tree. The relative lignin content of the stem was closely associated with peroxidase and coniferyl oxidase activity. A representative profile is shown (Fig. 5a-d). Phenolic content and coniferyl alcohol oxidase (a typically lignifying enzyme activity) showed a concomitant peaking with relative lignin content in all the trees though some variations in individual members from the same location were observed. NADH- oxidase and syringaldazine oxidase were found to be absent and coniferyl alcohol appeared to be the specific lignifying peroxidase when crude extracts were assayed.

Table 2a. Expression of substrate specific peroxidase (s) in crude extract.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Units activity/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-dianisidine</td>
<td>2.534 ± 0.8</td>
</tr>
<tr>
<td>Syringaldazine</td>
<td>not detectable</td>
</tr>
<tr>
<td>Coniferyl alcohol</td>
<td>12.84 ± 0.05</td>
</tr>
<tr>
<td>NADH</td>
<td>1.804±0.064</td>
</tr>
</tbody>
</table>

Table 2b. Expression of substrate specific peroxidase (s) in Sephadex G-50 of Eucalyptus sp.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Units activity/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-dianisidine</td>
<td>12.132 ± 0.435</td>
</tr>
<tr>
<td>Syringaldazine</td>
<td>4.506 ± 0.116</td>
</tr>
<tr>
<td>Coniferyl alcohol</td>
<td>14.409 ± 0.605</td>
</tr>
<tr>
<td>NADH</td>
<td>4.235±0.136</td>
</tr>
</tbody>
</table>

3.3. Gradational study

All the three lignification related parameters namely phenol content, peroxidase activity and lignin content, showed significant variations, with regard to both gradational and seasonal aspects (Fig 2a-2d). The following general trend was observed in all the three plants, from which time to time sampling was done for corresponding six months. Phenol content, peroxidase activity and lignin content (per gram dry weight, as well as per gram alcohol insoluble residue) were all found to be highest in the basal branch of each of the three plants, with gradual decline in the serially upper branches, being minimum in the apical branches. Simultaneously, every single branch showed a gradual increase in terms of Px activity, phenol...
content and lignin content steadily from January to May, although a sudden decrease in all of them was noticed in the month of June.

Table 3. Peroxidase activity in the seedlings of identified Eucalyptus plants

<table>
<thead>
<tr>
<th>Seedlings</th>
<th>Activity/g FW</th>
<th>Mother plant</th>
<th>Activity/g FW</th>
</tr>
</thead>
<tbody>
<tr>
<td>S5/1</td>
<td>0.7433</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S5/2</td>
<td>2.1946</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S5/3</td>
<td>2.9026</td>
<td>S5</td>
<td>5.68 ± 0.562</td>
</tr>
<tr>
<td>S5/4</td>
<td>1.5575</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S5/5</td>
<td>1.5570</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1/1</td>
<td>0.5663</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1/2</td>
<td>2.5309</td>
<td>S1</td>
<td>4.04 ± 0.322</td>
</tr>
<tr>
<td>S1/3</td>
<td>0.2530</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1/4</td>
<td>0.2831</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A3/1</td>
<td>0.1769</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A3/2</td>
<td>0.6831</td>
<td>A3</td>
<td>2.54 ± 0.149</td>
</tr>
<tr>
<td>A3/3</td>
<td>0.2530</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A3/4</td>
<td>0.1643</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A5/1</td>
<td>0.3142</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A5/2</td>
<td>0.2230</td>
<td>A5</td>
<td>3.65 ± 0.3321</td>
</tr>
<tr>
<td>A5/3</td>
<td>2.2830</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A5/4</td>
<td>0.4778</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

S: SAMBDIA, A: ARABARI

3.4. Purification

A summary of the result of the purification of Px is shown in Table 1. The crude extract contained Px at a specific activity of 1.852 ? mol orthodianisidine oxidized min⁻¹mg⁻¹ protein. Ammonium sulfate fractionation followed by Sephadex G-50 chromatography gave about 6-fold purification (sp.activity: 6.373). DEAE chromatography was an effective purification step. Here the entire activity was recovered without binding to the column material and gave about 67-fold purification (sp.activity: 66.51). The PAGE (Polyacrylamide gel electrophoresis) pattern indicated that this was a single enzyme.
Fig. 5. a-d. Seasonal variation in lignification associated parameters in Eucalyptus stem

5.a. Peroxidase

5.b. Phenol

5.c. Lignin

5.d. Coniferyl alcohol oxidase
Fig 3 & 4 shows the effect of pH and temperature on enzyme activity. The purified fraction showed a temperature optimum at 50°C. At 70°C the loss of Px activity was about 90% of its maximum value and with further increase of temperature the activity was completely lost. Similarly, at pH 7.5 approximately 82% of Px activity was lost and at pH 8.5 the activity was totally lost. The substrate specificity of this enzyme is shown in (Table 2a-b). Peroxidase activity in the crude extract utilized orthodianisidine (general substrate) and coniferyl alcohol but did not utilize syringaldazine the (typical substrate used to detect lignifying activity) (Table 2a). Further the impure preparation of Eucalyptus Px had very low to non-detectable NADH oxidase activity, which normally would supply H₂O₂ required for lignification, but the active fractions collected from gel exclusion chromatography could utilize all the substrates (Table 2b).

3.5. Correlation of isozyme pattern in trees and seedlings

The results on Px activity in selected seedlings (A3, A5, S5, and S1) are summarized in Table 3. In general, Px activity in seedlings tended to follow the trend in mother trees though there were a few exceptions. The extracts from the leaves of the selected seedlings were subjected to native PAGE and enzyme staining. Enzyme patterns did not differ between seedlings, neither did the bands differ from the mature trees. In both mature trees and seedlings the number of Px bands obtained were one major and one minor band.

3.6. Tissue culture

After some initial problems in adjusting the conditions of transfer and growth, we were able to produce shooting in several samples. Some of these shoots have now been transferred to rooting media. More samples are in the process of culture.

4. Discussion

4.1. Seasonal variation

It is apparent from Fig. 1 that Px activity was related with environmental temperature, showing lower values during winter and concomitant rise in activity with increase in temperature. In in vitro studies with cytoplasmic and cell wall Px of phloem and young xylem of *Populus sp*, both enzymic activities were maximum during the growing season (spring) and minimum activity was obtained in the dormant season (Imbery et al, 1985).
The apparent temperature sensitivity of Px, which was reflected by the variation in activity during the different seasons, might be related to growth and development of the plant. A sharp increase in Px activity in the growing season (autumn) was found in the needles of Norway spruce (Picea abies. L) (Polle et al, 1994).

4.2. Correlation of Px with lignin and its related parameters

The observation that higher Px activity is related to higher lignin content and vice-versa, is in line with the observation of Mader and Amberg – Fisher (1982), that plant Px catalyzes two separate reactions, that lead to polymerization of aromatic alcohol’s (namely p-coumaryl, coniferyl and sinapyl alcohols) into lignin.

The above observation was also supported by the study in many Pinus spp. where decreased Px activity was seen to be accompanied by reduced lignification (Cowles et al, 1989). The observation that higher phenol content was accompanied by higher lignin content and vice-versa, may be explained by the fact that phenols are precursors of monolignols viz. p-coumaryl, coniferyl and sinapyl alcohols (Polle et al. 1994).

4.3. Gradational study

A sharp increase in Px activity in the apical branches of all the three plants, than that of basal branches, during the later period of study may be due to better utilization of sunlight by those branches with increase in environmental temperature during the same period.

4.4. Purification

Separation of the Px using a combination of molecular sieving and ion exchange chromatography gave 66-fold purification. Gel studies indicated that this was a single enzyme. While the crude enzyme preparation and ammonium sulfate precipitated fractions were incapable of significantly utilizing NADH, the purified fractions could carry out this reaction. It appeared therefore, that the crude extract contained some inhibitory principle, which modulated the expression of the enzymic function. However oxidase activity could also be detected in purified portion. The finding supports the idea of some endogenous inhibitor or inhibitors in the plant extract, which was capable of preferentially blocking activity towards NADH and syringaldazine oxidation.

4.5. Correlation of isozyme pattern in mature trees and seedlings

The activity as well as the gel electrophoretic pattern of the seedling was compared in an effort to understand whether the expression of enzyme activity was transmitted.
to the seedlings and whether the latter could be categorized on this basis. It was
evident from the result that only one major and one minor band were obtained both
in CPTs as well in seedlings. The differences that emerged were at the level of
band intensity, indicating that, in the seedlings having lowerPx activity the
expression of the enzyme was affected rather than the nature of isozyme pattern.
In general we can say that expression of Px is a dominant phenotype and further
rapid screening of clonally propagated plants may be based on this parameter.

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with isolated cell wall – associated peroxides from cultured liverwort cells, Marchantia
polymorpha L. Plant cell Physiol. 28, 723-726.

complementary DNA encoding the lignin-forming Peroxidase from tobacco: molecular

Oxidation of nicotinamide dinucleotide and formation of hydrogen peroxide by cell wall


