Chemical characterization and spectroscopic analysis of the solubilization products from wheat straw produced by Streptomyces strains grown in solid-state fermentation

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INTRODUCTION

There is considerable interest in the biological degradation of lignocellulose in the environment and its potential for industrial application. The biotechnological potential of waste biomass bioconversion has centred upon the biological and chemical treatment of lignocellulosic material to produce a range of products, including ethanol and sugars (Hartley et al., 1987). Also, the transformation of wheat straw during the growth of micro-organisms under solid-state fermentation conditions has been examined for the upgrading of straw to produce pulps and animal feed (for a review see Dart & Betts, 1991). The potential role of lignocellulose-degrading enzymes in the bleaching of pulp for paper has been investigated as an alternative or supplement to chemical bleaching techniques, which cause environmental problems through the release of chlorine-containing effluents. Among the micro-organisms screened for the production of suitable enzymes, the actinomycetes are a group that have often been selected (Winter et al., 1991; Zhou et al., 1993).

Actinomycetes produce a range of hydrolytic and oxidative enzymes which act co-operatively in degradation of the lignocellulose complex during primary growth (Ball et al., 1990). The mechanisms involved in this degradation process have not yet been fully elucidated; however, the main role attributed to these micro-
organisms is solubilization rather than depolymerization and mineralization of lignin (McCarthy, 1987). The main product of solubilization of wheat straw and other lignocellulosic residues by actinomycetes is an acid-precipitable polymeric lignin (APPL; Crawford et al., 1983). This polymeric material is composed mainly of lignin but also contains carbohydrate, protein and ash (McCarthy, 1987). Previous studies have shown that the chemical composition of the APPL is dependent on the fermentation (submerged or solid-state) and on the method used for its extraction from culture supernatants (Borgmeyer & Crawford, 1985; Crawford et al., 1983).

A range of degradative techniques (acidolysis, mangenate oxidation, alkaline ester hydrolysis, thioacidolysis) (Crawford & Pometto, 1988) has been used along with non-degradative techniques (NMR and IR) (Seelenfreund et al., 1990; Crawford et al., 1983; McCarthy et al., 1986; Trigo & Ball, 1994) to analyse APPL composition. Cupric oxide degradation with GC–MS analysis is a degradative technique which cleaves β-O-4 links between the lignin groups and allows the quantification of cinnamic acids (Chang & Allen, 1971; Hedges & Ertel, 1982; González & Hedges, 1992). 13C NMR is a useful technique for analysing insoluble material, because it avoids the artefacts generated by degradative techniques (Fidalgo et al., 1993). In the case of straw lignin, additional problems arise from the structural complexity of the APPL through the presence of p-hydroxyphenyl, guaiacyl and syringyl units in association with cinnamic acids. There are several studies which establish differences between APPL harvested from different residues by different micro-organisms (Borgmeyer & Crawford, 1985; Trigo & Ball, 1994; McCarthy et al., 1986; Ramachandra et al., 1988; Ball et al., 1990), but it is important to consider the method used for obtaining lignin–carbohydrate complexes (APPL) from both inoculated and control samples. For APPL extraction from solid-state fermentation of corn stover lignocellulose, both water and NaOH have been used (Crawford & Pometto, 1988), and for this study we compared the extraction of APPL by these solvents, in order to assess the potential for using biological solubilization techniques, based on the use of streptomyces, in relation to chemical solubilization techniques. Both degradative (CuO oxidation) and non-degradative (IR and NMR) techniques were combined to study the solubilization of wheat straw during solid-state fermentation of straw by four Streptomyces strains.

**MATERIALS AND METHODS**

**Micro-organisms and growth conditions.** Three Streptomyces strains, Streptomyces chattanoogens UAH 23 and Streptomyces sp. UAH 52 and UAH 33 were selected in our laboratory for their ability to degrade lignocellulosic residues. Streptomyces viridosporus strain T7A, a well-characterized lignocellulose-degrading actinomycete (Crawford et al., 1983) was used, as a control throughout. All the strains were grown on GAE agar, containing (g l–1): glucose, 10 g; L-asparagine, 1 g; yeast extract (Difco), 0.5 g; K2HPO4, 0.5 g; MgSO4.7H2O, 0.5 g; FeSO4.7H2O, 0.001 g. Streptomyces viridosporus, and strains UAH 23 and UAH 52 were incubated at 28 °C, and strain UAH 33 at 37 °C. The strains were stored as spore suspensions in 20% (v/v) glycerol at –20 °C.

For experiments, spores were harvested from GAE agar dishes with distilled water containing Tween 80 (0.01%), and a standard spore suspension (5 ml, 107 c.f.u. ml–1) was used to inoculate flasks containing 150 ml of mineral basal medium (MBM; Crawford, 1978) supplemented with 0.6% yeast extract.

Wheat straw (Triticum aestivum var. maestro) was ground in a Janke and Kunkel A-10 mill to pass through a 40-mesh screen and air-dried for 24 h at 50 °C. To facilitate the colonization of the substrate unplugged 2 l flasks containing 10 g wheat straw were steamed for 1 h (Berrocal et al., 1996). The flasks were plugged with cotton stoppers and autoclaved for 20 min at 120 °C.

Cultures were incubated for 48 h with shaking at 200 r.p.m. and the mycelia harvested by centrifugation (10000 g, 15 min). For optimal solid-substrate fermentation conditions the mycelium from each flask was resuspended in 50 ml MBM and then used to inoculate wheat straw (10 g) (Berrocal et al., 1996). Cultures were incubated statically for 28 d. Uninoculated controls were incubated as above.

**Extraction of APPL.** APPL was extracted from solid-substrate fermentation products after 28 d growth by the addition of 400 ml NaOH (0.1 M) or distilled water. Samples were then steamed at 100 °C for 1 h, filtered through Whatman no. 54 filter paper and washed again with 400 ml NaOH (0.1 M) or distilled water at 60 °C. Supernatants were acidified with HCl (12 M) to pH 1–2 and the APPL harvested by centrifugation (12000 g, 10 min). Finally, the APPL was washed twice with distilled water (pH 5) and freeze-dried.

Yield of APPL was expressed as a percentage of the freeze-dried APPL per g wheat straw. Klassen lignin was estimated as the ash-free residue after Saeman’s hydrolysis of APPL (Efland, 1977). Ash content was determined after treatment at 575 °C for 6 h.

**Cupric oxide degradation.** Samples (100 g) of APPL obtained by NaOH or distilled water extraction were maintained at 180 °C for 3 h in nitrogen Teflon bombs containing CuO (2 g), Fe(NH4)2(SO4)2·6H2O (200 mg) and 14 ml boiled NaOH (2 M). After precipitation with HCl (12 M), degradation products were recovered with 3 vols diethyl ether (20 ml) and dried under nitrogen. Pyridine (100 ml) was added to the dried residues and 25 ml derivatized (silylated) with 40 μl bis(trimethylsilyl)-trifluoroacetamide (BSTFA). The mixture was heated at 60 °C for 10 min with periodic shaking to dissolve residues. Chromatographic analyses of APPL were carried out in a Perkin-Elmer Sigma 3B gas chromatograph, coupled to an electron-impact mass detector (ITD). Trimethylsilyl derivatives were separated in a SPB-1 capillary column (Supelco 0.25 mm stationary-phase thickness, 30 m×0.25 mm i.d.). Nitrogen was used as carrier gas. Initial column oven temperature was 100 °C; the temperature was increased at 4 °C min–1 to a final value of 270 °C.

Quantifications were based on the area of the internal standard (ethyl vanillone) and the response factors obtained from standard compounds (three p-hydroxyphenyls (p-hydroxybenzaldehyde, p-hydroxyacetophenone, p-hydroxybenzoic acid); three guaiacyl compounds (vanillin, acetovanillone and vanillic acid); three syringyl compounds (syringaldehyde, acetoxy-syringone and syringic acid); trans-ferulic acid and p-
Results are the means of triplicate assays ± SD.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Extraction...</th>
<th>Yield [mg (g wheat straw)⁻¹]</th>
<th>Klaason lignin (%)*</th>
<th>Ash (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NaOH</td>
<td>Water</td>
<td>NaOH</td>
</tr>
<tr>
<td>Uninoculated control</td>
<td>68 ± 0.5</td>
<td>6 ± 0.5</td>
<td>4.73 ± 0.03</td>
<td>0.37 ± 0.01</td>
</tr>
<tr>
<td>Streptomyces UAH 23</td>
<td>163 ± 14</td>
<td>20 ± 1</td>
<td>13.49 ± 0.19</td>
<td>1.69 ± 0.05</td>
</tr>
<tr>
<td>Streptomyces UAH 33</td>
<td>142 ± 14</td>
<td>23 ± 2</td>
<td>11.83 ± 0.17</td>
<td>1.76 ± 0.06</td>
</tr>
<tr>
<td>Streptomyces UAH 52</td>
<td>143 ± 15</td>
<td>21 ± 1.8</td>
<td>11.46 ± 0.17</td>
<td>1.56 ± 0.05</td>
</tr>
<tr>
<td>S. viridusporus</td>
<td>141 ± 16</td>
<td>9 ± 0.9</td>
<td>11.60 ± 0.15</td>
<td>0.66 ± 0.02</td>
</tr>
</tbody>
</table>

* Results are expressed as percentage of total Klason lignin in wheat straw (24.5 ± 0.05%).
† Results are expressed as percentage of total ash in wheat straw (2.29 ± 0.07%).
strain 33 showed the lowest ratio (1:3, Table 2), indicating degradation of the syringyl moieties by this strain. The low proportion of H units detected in all APPL samples extracted with NaOH suggests that many of the H-lignin bonds are esters which are saponified during extraction with NaOH (Fidalgo et al., 1993). The ferulic acid content of APPL extracted with NaOH was always greater than the coumaric acid content, with the highest values detected in APPL from S. chattanoogensis strain UAH 23 (17.5 and 5.5% for ferulic and coumaric acid respectively). Analyses of undegraded wheat straw have shown that the concentration of

<table>
<thead>
<tr>
<th>Extraction</th>
<th>APPL characteristic</th>
<th>Inoculum:</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Control*</td>
</tr>
<tr>
<td>S/G</td>
<td>p-Coumaric†</td>
<td>1:9</td>
</tr>
<tr>
<td></td>
<td>trans-Ferulic†</td>
<td>14:3</td>
</tr>
<tr>
<td>S/G</td>
<td>p-Coumaric†</td>
<td>1:6</td>
</tr>
<tr>
<td></td>
<td>trans-Ferulic†</td>
<td>3:9</td>
</tr>
</tbody>
</table>

* Uninoculated wheat straw extracted with distilled water and NaOH.
† Mol per 100 mol lignin etherified (H + G + S).
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IR spectroscopy

IR spectra of APPL from inoculated wheat straw and the un inoculated control using water are presented in Fig. 2. The assignments of the most characteristic IR signals have been made in accordance with previous studies (Scalbert *et al.*, 1985; Xiao-An *et al.*, 1989; Fidalgo *et al.*, 1993). The spectra are generally similar, with the most prominent bands corresponding to lignin (1600, 1510, 1460, 1425, 1335 and 1270 cm⁻¹). Carbohydrate signals were detected at 1380 and 1040 cm⁻¹, although no differences in these signals could be detected in APPL from inoculated and control spectra (Fig. 2). Bands from carbonyl groups were present at 1720 cm⁻¹ (non-conjugated carbonyl groups, e.g. aliphatic acids, cinnamic acids and fatty acids) and 1660 cm⁻¹ (conjugated carbonyl groups of amides) (Fengel & Wegener, 1984). The broad band between 1620 and 1600 cm⁻¹, assigned to the tension of the aromatic rings, is more intense in the APPL extracted from straw inoculated with *Streptomyces* strains UAH 23, UAH 33 and UAH 52, reflecting the greater lignin content of the APPL from these strains. The decrease in the intensity of the signals at 1510 and 1460 cm⁻¹ in the IR spectra from APPL extracted from cultures of *S. viridosporus* and *S. chattanoogensis* UAH 23 suggests a loss of aryl radicals in the lignin side-chain,

![Fig. 2. IR spectra of APPL extracted with water from wheat straw inoculated with *Streptomyces* strains. Control is uninoculated wheat straw extracted with distilled water.](image)

(S/G = 1.0, Table 2). The highest ferulic acid concentrations were detected in APPL from *Streptomyces* strain UAH 52 (15.6 and 10.9 % respectively, Table 2). The increased concentration of coumaric acid, but not ferulic acid, in APPL extracted with water in comparison to NaOH-extracted APPL confirms the suggestion that coumaric acid in wheat straw is mainly ester-linked to lignin while ferulic acid is mainly ether-linked (Scalbert *et al.*, 1985; Iiyama *et al.*, 1990). Saponification of these ester links occurs during NaOH extraction leaving only the ether links, thereby decreasing the amount of coumaric acid recovered (Billa & Monties, 1995). In contrast, ferulic acid complexes are mainly bound to the lignin in cell walls of wheat straw via ether links, and as these bonds are not disrupted by extraction with NaOH, the concentration of ferulic acid detected was greater (Table 2). The increase in coumaric acid, and perhaps to a lesser extent ferulic acid, in APPL extracted with water from inoculated wheat straw when compared to the control APPL may be as a consequence of the action of phenolic acid esterases, enzymes which have been detected in culture supernatants from streptomycetes grown on media containing wheat straw (Deobald & Crawford, 1987; Donnelly & Crawford, 1988; Christov & Prior, 1993). Alternatively, the increase in ferulic acid detected in APPL extracted with water from inoculated straw may be due to the formation of ferulic acid complexes which prevent extraction with water (but not NaOH) from un inoculated straw (Jeffries, 1990). However, during microbial solubilization, the ester links of the ferulic acid could be cleaved, perhaps by ferulic acid esterase (Christov & Prior, 1993) thus enabling the ferulic acid, which remains ether linked to the lignin, to be solubilized.

ferulic acid is generally higher than the concentration of coumaric acid (Hartley, 1972; Mueller-Harvey *et al.*, 1986; Iiyama *et al.*, 1990). In addition, ferulic acid may be cross-linked between lignin and hemicellulose by esterification of its carboxyl groups with the hydroxy groups of the arabinose residue in hemicellulose and by etherification of its phenolic hydroxyl groups with phenyl hydroxyls of lignin (Jeffries, 1990). Enzyme activities produced by streptomycetes involved in the cleavage of ether linkages (Ramachandra *et al.*, 1988) or ester linkages (Christov & Prior, 1993) in lignocellulose residues have been described. The difference between the ferulic acid content of the APPL extracted with water from inoculated and uninoculated straw may be attributed to the production of these activities by the streptomycetes studied.

Comparing the molar H:G:S relationship between APPL extracted with distilled water and NaOH, p-hydroxyphenyl groups were much higher in APPL extracted with water, confirming that these units are mainly ester-linked to lignin (Fidalgo *et al.*, 1993). The S/G ratio of APPL from inoculated straw was always lower than that of the APPL extracted from the control straw, with APPL from *S. viridosporus* and *S. chattanoogensis* UAH 23 exhibiting the lowest S/G values
perhaps as a consequence of microbial activity (Almendros et al., 1992).

The differences between signals corresponding to 1510, 1460 and 1335 cm\(^{-1}\) in the spectra of APPL extracted from wheat straw inoculated with \textit{S. chattanoogensis} UAH 23 and \textit{S. viridosporus} correspond to alterations in the lignin structure, mainly in the S units, supporting the data obtained by CuO (Table 2). IR spectra of APPL extracted from control and inoculated wheat straw extracted with NaOH were poorly defined, probably due to the effects of NaOH on the ester linkages between the main structural moieties of wheat straw (data not shown).

### Solid-state CPMAS \(^{13}\text{C}\) NMR

NMR spectra of the APPL from control and inoculated wheat straw extracted with NaOH and water are shown in Fig. 3 and Fig. 4 respectively. Integration of spectral areas corresponding to different carbon types in the CPMAS \(^{13}\text{C}\) NMR spectra are shown in Table 3. Because of the similarity between spectra obtained from APPL extracted from wheat straw inoculated with Streptomyces strains UAH 52 and UAH 33, only spectra for strain UAH 52 are presented in Fig. 3. The assignments of the most prominent NMR signals have been made in accordance with previous studies (Lüdemann & Nimz, 1973; Maciel et al., 1985; Scalbert et al., 1986; Almendros et al., 1992).

For lignin, 153 p.p.m. corresponds to the \(C_2\) and \(C_5\) in syringyl groups (S-etherified), 148 p.p.m. indicates the \(C_2\) and \(C_5\) in guaiacyl (G) units (etherified) and 146 p.p.m. corresponds to the \(C_2\) and \(C_5\) in S (phenolic) groups; 133 p.p.m. indicates \(C_1\) and \(C_8\) in S and \(C_2\) in G, 128 p.p.m. indicates the \(C_4\) and \(C_8\) groups in \(p\)-hydroxyphenyl (H), 105 p.p.m. corresponds to \(C_2\) and \(C_8\) in S, 84 p.p.m. indicates the \(C_8\) in \(\beta-O-4\), and 55 p.p.m. corresponds to methoxy C. Several signals correspond to polysaccharide carbon units, 75 p.p.m. for \(C_3\), \(C_2\) and \(C_5\). Two other signals were present, 172 p.p.m. for carbonyl C and 31 p.p.m. for non-substituted alkyl C. The signal at 21 p.p.m. corresponds to acetyl C from hemicellulose.

In NMR spectra for APPL extracted from inoculated wheat straw with NaOH, the signal at 31 p.p.m. (non-substituted alkyl C) showed a decreased intensity compared with the signal from the uninoculated control. In all spectra the signal at 153 p.p.m., corresponding to \(C_2\) and \(C_5\) in S, was greater than the signal at 148 p.p.m., corresponding to the \(C_3\) and \(C_4\) signal in G. This confirms the data obtained from CuO oxidation of APPL indicating that the concentration of S units is always greater than the concentration of G units. No significant differences in the intensity of signals between 200 and 160 p.p.m. and between 21 and 0 p.p.m. could be detected in APPL extracted from inoculated straw, possibly due to cleavage of the ester links during NaOH extraction of APPL. The low H concentration of NaOH extracted APPL was also confirmed by NMR with the appearance of the signal at 128 p.p.m. as only a shoulder (Fig. 3). No differences in the signal obtained at 55 p.p.m. (corresponding to methoxy groups) could be detected between APPL from control and inoculated straw, suggesting that these strains do not demethylate lignin during growth on straw.

In NMR spectra of APPL from inoculated straw extracted with water, the signal corresponding to the carbonyl groups (172 p.p.m.) was decreased with respect to the spectra of the control APPL. However in a study by McCarthy et al. (1986) an increase in the signal at 172 p.p.m. of APPL extracted from straw after 14 d growth in submerged culture of \textit{Thermomonospora mesophila} was observed. This increase in signal intensity was explained in terms of the ability of \textit{T. mesophila} to oxidise the \(\alpha\)-carbons in the lignin propanoid chains, a reaction which would be expected to improve lignin solubility (McCarthy et al., 1986). Nevertheless, it is...
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Fig. 4. CPMAS $^{13}$C NMR spectra of APPL extracted with water from wheat straw inoculated with Streptomyces strains. Control is uninoculated wheat straw extracted with distilled water.

Table 3. Carbon composition from integration of CPMAS $^{13}$C NMR spectra of controls and APPL extracted with NaOH and water from wheat straw inoculated with Streptomyces strains

<table>
<thead>
<tr>
<th>Extraction</th>
<th>Inoculum</th>
<th>Carboxyl C (210–160 p.p.m.)</th>
<th>Aromatic C (160–110 p.p.m.)</th>
<th>O-Alkyl C* (110–46 p.p.m.)</th>
<th>Alkyl C (46–0 p.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH</td>
<td>Control†</td>
<td>10.60</td>
<td>37.00</td>
<td>42.40</td>
<td>10.00</td>
</tr>
<tr>
<td></td>
<td>UAH 23</td>
<td>7.00</td>
<td>40.80</td>
<td>44.00</td>
<td>8.00</td>
</tr>
<tr>
<td></td>
<td>UAH 33</td>
<td>10.00</td>
<td>38.40</td>
<td>43.80</td>
<td>7.80</td>
</tr>
<tr>
<td></td>
<td>UAH 52</td>
<td>11.90</td>
<td>39.20</td>
<td>41.20</td>
<td>7.70</td>
</tr>
<tr>
<td></td>
<td>S. viridosporus</td>
<td>8.60</td>
<td>40.00</td>
<td>43.60</td>
<td>7.80</td>
</tr>
<tr>
<td>Water</td>
<td>Control†</td>
<td>24.18</td>
<td>17.63</td>
<td>27.24</td>
<td>30.93</td>
</tr>
<tr>
<td></td>
<td>UAH 23</td>
<td>7.00</td>
<td>40.00</td>
<td>43.70</td>
<td>9.30</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>S. viridosporus</td>
<td>11.30</td>
<td>37.30</td>
<td>38.30</td>
<td>13.00</td>
</tr>
</tbody>
</table>

* Oxygen-linked aliphatic C, including lignin methoxy groups.
† Uninoculated wheat straw extracted with distilled water and NaOH.

Changes in the signals at 153 p.p.m. (corresponding to the S units) and at 148 p.p.m. (G) in the spectrum from APPL obtained from inoculated wheat straw correspond to changes in the S/G ratio as determined by CuO oxidation. The increased concentration of H units in the APPL extracted with water compared with APPL extracted with NaOH was clearly evident in the signal intensity at 128 p.p.m. No differences in the intensity of the signal at 55 p.p.m. could be detected in APPL extracted with water, confirming the results obtained by McCarthy et al. (1986) with Thermomonospora mesophila.

One limitation in the use of NMR spectra is the inability to distinguish between the hemicellulose and cellulosic components of APPL because of the overlap between resonance signals (Gamble et al., 1994). However, in the
110–46 p.p.m. region, significant modifications can be detected in APPL from inoculated straw. These modifications confirm the ability of streptomycetes to carry out simultaneous degradation of all the three main components of lignocellulose.

Concluding remarks
The complementary techniques used in this study allow the fullest interpretation of the results. NMR and IR allow the analysis of a relatively unaltered substrate. However, the complexity of APPL inevitably means that signals relating to different components of APPL overlap, complicating interpretation. CuO degradation signals relating to different components of APPL obtained through NaOH extraction is a relatively mild procedure for analysing straw lignin since it allows the estimation by GC–MS analysis of lignin units and cinnamic acids separately.

The results from the analysis of APPL using destructive and non-destructive techniques show that the properties of the APPL extracted by water and NaOH are very different. APPL obtained through NaOH extraction results in greater yields of the lignin part of the complex, but causes modifications in the APPL. The most significant alteration can be detected in the cinnamic acid and the aromatic moiety of lignin. The breaking of the ester links between hemicellulose and lignin and between hemicellulose and cinnamic acids decreases the number of alkyl and carbonyl groups present. The cleavage of these ester cross-linking groups by NaOH extraction enables the lignin to be more readily solubilized from the hemicellulose fraction. When APPL was extracted with water, ester links remain, allowing a better assessment of the process of solubilization by streptomycetes. The possible involvement of esterases and peroxidases in the lignocellulose solubilization could only be envisaged when water was used to harvest APPL.

We conclude through the study of APPL extracted with water that streptomycetes are able to modify the aromatic moiety of lignin during the solubilization process, with modifications occurring in the syringyl moieties. In addition the ability of streptomycetes to break the ester bond between cinnamic acids and hemicellulose can be inferred. However, for non-biological studies, such as chemical processes, the use of NaOH for lignin solubilization (as is used in the paper and pulp industry) represents a more efficient process as higher APPL yields are obtained when compared to APPL extracted with water. The increased yields of APPL obtained when straw is first incubated with streptomycetes, followed by extraction with NaOH (0.1 M), suggest a possible role for these organisms or their enzymes to enhance alkali extractions in the industrial solubilization of lignocellulose.

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