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Colour pigments of *Trichoderma harzianum* Preliminary investigations with thin-layer chromatography–Fourier transform infrared spectroscopy and high-performance liquid chromatography with diode array and mass spectrometric detection

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Abstract

The colour pigments of *Trichoderma harzianum* fermentation broth were separated and the main fractions were tentatively identified by reversed-phase thin-layer chromatography–Fourier transform infrared spectroscopy (RP-TLC–FT-IR), RP-HPLC–diode array detection and RP-HPLC–MS. It was established that the multistep gradient elution developed for RP-TLC separation of pigments can be successfully used as a pilot method for the rational design of gradient elution in RP-HPLC for the separation of the same pigments. FT-IR and MS measurements were unable to identify the exact chemical structures of the main pigment fractions, the presence of OH, =CH and C=O (RP-TLC–FT-IR) and OH and NH_2 substructures (RP-HPLC–MS) was confirmed. It was assumed that the main pigment fractions are oxidation polymers originating from monomer molecules containing polar substructures and double bonds in the alkyl chain which are liable for oxidation during the aerobic fermentation process.

Keywords: *Trichoderma harzianum*; Pigments

1. Introduction

Various chromatographic methods have been extensively used for the separation and quantitative determination of a wide variety of organic and inorganic components in microorganisms [1]. The more profound knowledge of the character and quantity of molecules present in microorganisms facilitates the understanding of the biochemical procedures exploited in various biotechnological processes, advances the development of molecular biology and may help in the identification of the microorganism [2]. As some microorganisms produce characteristic colour pigments, the separation and quantitative determination of these pigments may also help their identification. Pigments are nonvolatile and are easily decomposed at higher temperatures, therefore, they can be analyzed only with liquid chromatographic methods such as thin-layer (TLC) and high-performance liquid chromatography (HPLC).

Because of its advantageous properties (no need for complicated instrumentation, manyfold possibilities of detection) TLC has been frequently applied for the separation and quantitative or semi-quantitative determination of natural pigments. Previous results in the employment of TLC in the analysis of

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natural pigments in general [3], and especially in plants [4] have been reviewed.

Theoretical studies established that the rapid and inexpensive TLC can be successfully used not only as an independent separation technique but also as a pilot method for HPLC [5]. This finding also has considerable practical importance, because TLC as a pilot method for HPLC not only facilitates the determination of an appropriate mobile phase for HPLC but also enables detection of compounds from the sample matrix that do not migrate or which show negligible mobility with mobile phase under investigation [6]. The inherent disadvantages of traditional TLC is that developments can be carried out only under isocratic conditions resulting in lower separation capacity compared with gradient elution in HPLC. To overcome this difficulty and to increase the efficacy of TLC multistep gradient elution was developed [7,8]. Two different modes of multistep gradient elution can be employed: stepwise mobile phase gradient elution [9] and programmed multiple development or its automated version [10]. Multistep gradient elution was optimized for two-step [11] and multistep development [12]. It has been recently found that the use of multistep gradient elution in TLC as a pilot method may facilitate the development of gradient elution even in HPLC [13].

Traditional chromatographic methods are not suitable for the identification of unknown compounds, the coelution of a standard compound with the unknown solute is not an exact proof of the identity of the two molecules. The application of hyphenated techniques such as TLC coupled to Fourier transform infrared spectroscopy (FT-IR), HPLC with diode array (DAD) and mass spectrometric (MS) detection overcomes this difficulty making possible the identification of solutes without an authentic standard. TLC–FT-IR has been successfully used for the in situ identification of solutes separated on TLC plates [14,15]. As TLC sorbents are strong absorbers of infrared light the spectra of solutes sometimes partially or entirely coincides with the spectra of support. In these instances the solutes have to be extracted from the plates for better identification.

Because of the considerable biological activity of compounds produced by *Trichoderma harzianum* [16,17] many chromatographic methods such as gas chromatography [18] and HPLC [19] have been employed for the separation and quantitation of its constituents. The objectives of the study were the separation and quantitative determination of the colour pigments of *Trichoderma harzianum* by reversed-phase HPLC (RP-HPLC) using RP-TLC as pilot method and the use of TLC–FT-IR, HPLC–DAD and HPLC–MS for the tentative identification of the main fractions. The investigations were motivated by the supposition that the measurement of the quantity and distribution of colour pigment of *Trichoderma harzianum* may contribute to its chemotaxonomic evaluation [20].

To the best of our knowledge the colour pigments of *Trichoderma harzianum* have never been studied in detail and the pigments have never been separated and tentatively identified by hyphenated chromatographic techniques.

2. Materials and methods

Trichoderma harzianum 2424 (University of Salamanca, Faculty of Medicine, Institute of Microbiology and Biochemistry, Salamanca, Spain) was fermented in 200 g/l Potato Dextrose Broth (DIFCO, Franklin Lakes, NJ, USA) containing 20 g/l Bacto Dextrose. Fermentation was carried out at 25°C for 72 h on a rotary shaker (150 rpm). After fermentation 400 ml of fermentation broth was extracted with 400 ml of ethyl acetate. The organic fraction was separated and evaporated to dryness in a vacuum evaporator (Büchi B-171, Laboratory Devices, Ontario, Canada). Sample was stored at 0°C in a nitrogen atmosphere.

2.1. RP-TLC

An aliquot of 3 mg of the yellow pigments from the fermentation broth of *Trichoderma harzianum* was dissolved in 1 ml of methanol and was used as sample solution for RP-TLC. Aliquots of 5 μl were spotted onto Alugram RP-18W/UV_{254} plates (Macherey–Nagel, Düren, Germany). Developments were carried out in sandwich chambers (22×22×3 cm) at ambient temperature (20±2°C). Various mixtures of water and acetone were employed as mobile phases the acetone concentration ranging from 25 to 90% (v/v). As isocratic separations were ineffective at each concentration of the organic modifier, two-step gradient elutions were applied. The composition of

the mobile phase for the first step was always water–acetone (1:9, v/v) (separation distance 3 cm), the concentration of acetone in the eluent was 55, 60, 65 and 70% (v/v) for the second step (separation distance 13 cm). It is well known that the majority of separations of pigments in TLC have been carried out in adsorption separation mode. The use of RP-TLC was motivated by the consideration that HPLC determination of pigments is generally performed in RP mode and only RP-TLC is suitable as a pilot method for the rational design of a RP-HPLC procedure for pigment separation. Because of the possible sensitivity of pigments to light, development was performed in the dark. After development the plates were dried at room temperature, evaluated visually and the plates showing good separation evaluated by the Shimadzu CS-930 dual-wavelength TLC scanner (Kyoto, Japan) at 400 nm. The intra-day reproducibility was determined by five independent parallel measurements, the inter-day reproducibility was calculated from five parallel measurements performed on each day for five days. The standard deviations of inter- and intra-day reproducibilities were compared by using the ''F'' probe. The mean values of peak area and R_F values and the corresponding relative standard deviations (RSDs) were calculated. The calculation of the RSD values was motivated by the fact that the RSD contains valuable information on the reproducibility and repeatability of the chromatographic system and the separation method applied. The two main fractions were evaluated by in situ FT-IR (Nicolet 170SX FT-IR spectrometer, Madison, WI, USA, equipped with a diffuse reflectance accessory prepared in our institute). As the support interfered considerably with the FTIR spectra the two fractions were scraped off the layer and placed in a glass tube of 100×10 mm. Pigments were removed by washing the tube with 20 ml of acetone at a flow-rate of ca. 0.5 ml/min. The eluate was concentrated in vacuum, redissolved in 20 μl acetone, and investigated by the traditional FT-IR method (Nicolet Magna 750 FT-IR spectrometer) using a KBr pellet of 4 mm diameter; resolution 4 cm^{-1}; scan 128) the spectra was tentatively identified by using an FT-IR spectra library [21].

2.2. HPLC–DAD

Measurements were performed with an ISCO (Lincoln, NE, USA) Model 2360 pump, a Waters (Milford, MA, USA) 991 photodiode-array detector, an NEC PowerMate SX/16 computer with a DAD program, and a Valco (Houston, TX, USA) injector with a 20-μl loop. Separations were carried out on a 250 mm×4 mm I.D. column (Hypersil C_{18}, particle size 5 μm, Phenomenex, CA, USA). Detection wavelength was between 320 and 450 nm. The gradient consisted of water–acetone mixtures: initial acetone concentration 5% for 5 min, increased to 67% in 25 min, increased to 94% in 15 min, final hold, 40 min. Flow-rate was 1 ml/min. Column was not thermostated, each separation was made at ambient temperature (20±2°C). Sample prepared for TLC was diluted tenfold and used for HPLC–DAD. As it cannot be excluded that one or more of the yellow pigments produced by *Trichoderma harzianum* are similar to the carotenoids of paprika (*Capsicum annuum*) [22] the pigments extracted from paprika powders [23] were separated with the same RP-HPLC–DAD method, and the visible spectra of the peaks showing similar retention times were compared. Validation parameters (intra- and inter-day reproducibility) were determined as described above.

2.3. HPLC–MS

The chromatographic system was the same as for HPLC–DAD. Detection was performed with a Perkin-Elmer Sciex API 165 mass spectrometer with atmospheric pressure chemical ionization (APCI) heated nebulizer ion source interface operated in the positive mode. The corona discharge needle was set to 5 μA and the orifice potential was 20. The quartz tube temperature was 200°C. Dry nitrogen was used as the nebulizing and curtain gas. Full scan acquisitions (100–650 u) were performed, the cycle time being 2 s.

3. Results and discussion

3.1. RP-TLC

Each isocratic development was unsuitable for the separation of all pigment fractions. A considerable quantity of pigments remained on the start at lower concentrations of acetone which can be removed only at 90% (v/v) of acetone. However, at this high

concentration of organic modifier the less lipophilic fractions migrated with the eluent front making separation impossible. The number and R_F value of pigment fraction markedly depended on the concentration of the organic modifier in the mobile phase. Each pigment showed regular retention behaviour on each plate the retention decreased with increasing concentration of organic component in the mobile phase.

The best separation of the colour pigments was obtained by the two-step gradient development using water–acetone (45:55, v/v) in the second step (Fig. 1). However, the densitogram clearly shows that the separation of colour pigments is incomplete even in the best elution system. This result may be due to the high number of fractions in the sample and the inherent low theoretical plate number of TLC even with multistep gradient elution. The retention factors (R_F values), peak areas % (according to the densitogram obtained at 400 nm) and RSDs of intra- and inter-day reproducibility are compiled in Tables 1 and 2. The validation parameters are similar to those generally achieved in TLC and they neither depend on the R_F value nor on the peak area of the individual pigment fractions. However, the RSD values were higher for peak areas than for R_F values. This discrepancy can be explained by the fact that pigments were not baseline separated in RP-TLC

Table 1
Retention factor (R_F) of pigment fractions of *Trichoderma harzianum* separated with multistep gradient elution in RP-TLC and inter- and intra-day reproducibility

Peak No.	R_F	Reproducibility (RSD, %)	
		Intra-day	Inter-day
1	0.06	4.21	4.72
2	0.13	4.44	4.59
3	0.26	3.92	4.27
4	0.77	4.57	5.01
5	0.86	4.10	4.34
6	0.92	4.34	4.57
7	0.96	4.92	5.27

Table 2
Relative peak areas (%) of pigment fractions of *Trichoderma harzianum* separated with multistep gradient elution in RP-TLC and inter- and intra-day reproducibility

Peak No.	Peak area	Reproducibility (RSD, %)	
		Intra-day	Inter-day
1	19.79	4.35	4.84
2	8.87	6.12	6.56
3	14.87	4.21	4.45
4	43.43	4.02	4.65
5	6.88	6.97	5.30
6	4.91	6.53	6.88
7	1.25	6.92	5.41

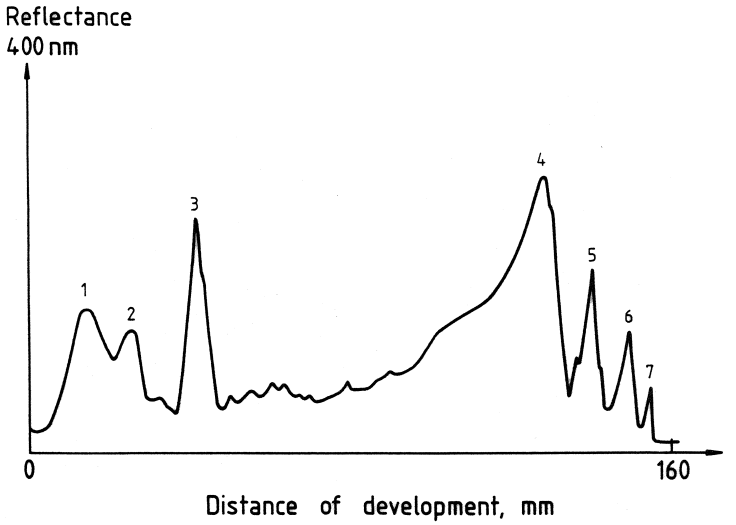

Fig. 1. Densitogram of colour pigments of *Trichoderma harzianum*. Alugram RP-18W/UV_{254} plates. Gradient: water–acetone (10:90, v/v) for 3 cm; water–acetone (45:55, v/v) for 13 cm. Detection wavelength 400 nm.

which increased the inaccuracy of integration. No significant differences were found between the standard deviations of intra- and inter-day reproducibilities indicating the acceptable reproducibility of TLC system.

FT-IR spectra of the two main fractions extracted from the plates and the spectra of monoelaidin selected by the library program are shown in Fig. 2. The facts that the monoelaidin is white and the match is only 81.65% indicate the inadequate identification of pigments. The spectra of the two main fractions are similar suggesting the close relationship between these two pigment fractions. The strong OH, =CH and C=O vibrations found in monoelaidin and in the TLC extracts unambiguously proves the presence of oxygen and unsaturated substructures in the pigment molecules.

3.2. HPLC–DAD

Typical chromatograms of pigments extracted from *Trichoderma harzianum* fermentation broth and paprika (*Capsicum annuum*) powders are shown in Fig. 3A and B, respectively. The chromatograms clearly show that *Trichoderma harzianum* produces a wide variety of pigments which differ considerably in lipophilicity. The retention times of some pigments are very similar to those of pigments extracted from paprika powder (i.e., fractions 1, 2, 5, 8 and 11 on the chromatogram of the pigments of *Trichoderma harzianum*). However, the comparison of the visible spectra of *Trichoderma harzianum* and paprika pigments showing similar retention times with DAD clearly show that the spectra are different. Colour pigments extracted from paprika show a maximum absorbance in the visible region, while pigments of *Trichoderma harzianum* show an increasing absorbance towards the UV region without a maximum in the wavelength range under investigation.

This finding indicates that the chemical structures of carotenoids in paprika and those of pigments produces by *Trichoderma harzianum* are different. Because of the high number of pigment fractions it can be assumed that besides other well established microbiological methods the pigment profile obtained by RP-HPLC can help in the identification of *Trichoderma harzianum.* The chromatographic and

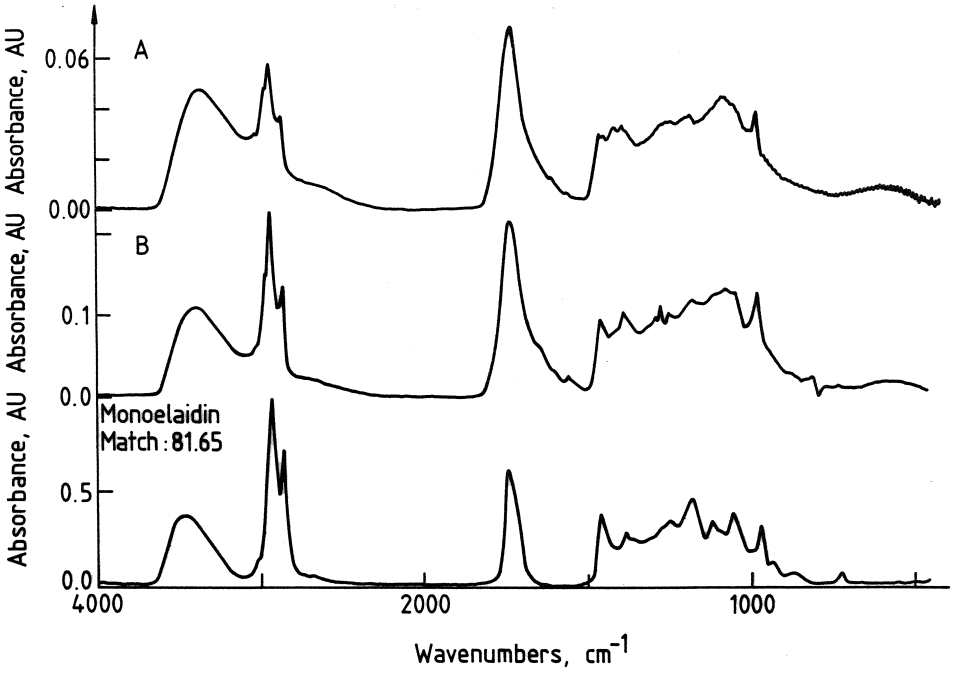

Fig. 2. FT-IR spectra of two main pigment fractions of *Trichoderma harzianum* separated by RP-TLC and of monoelaidin.

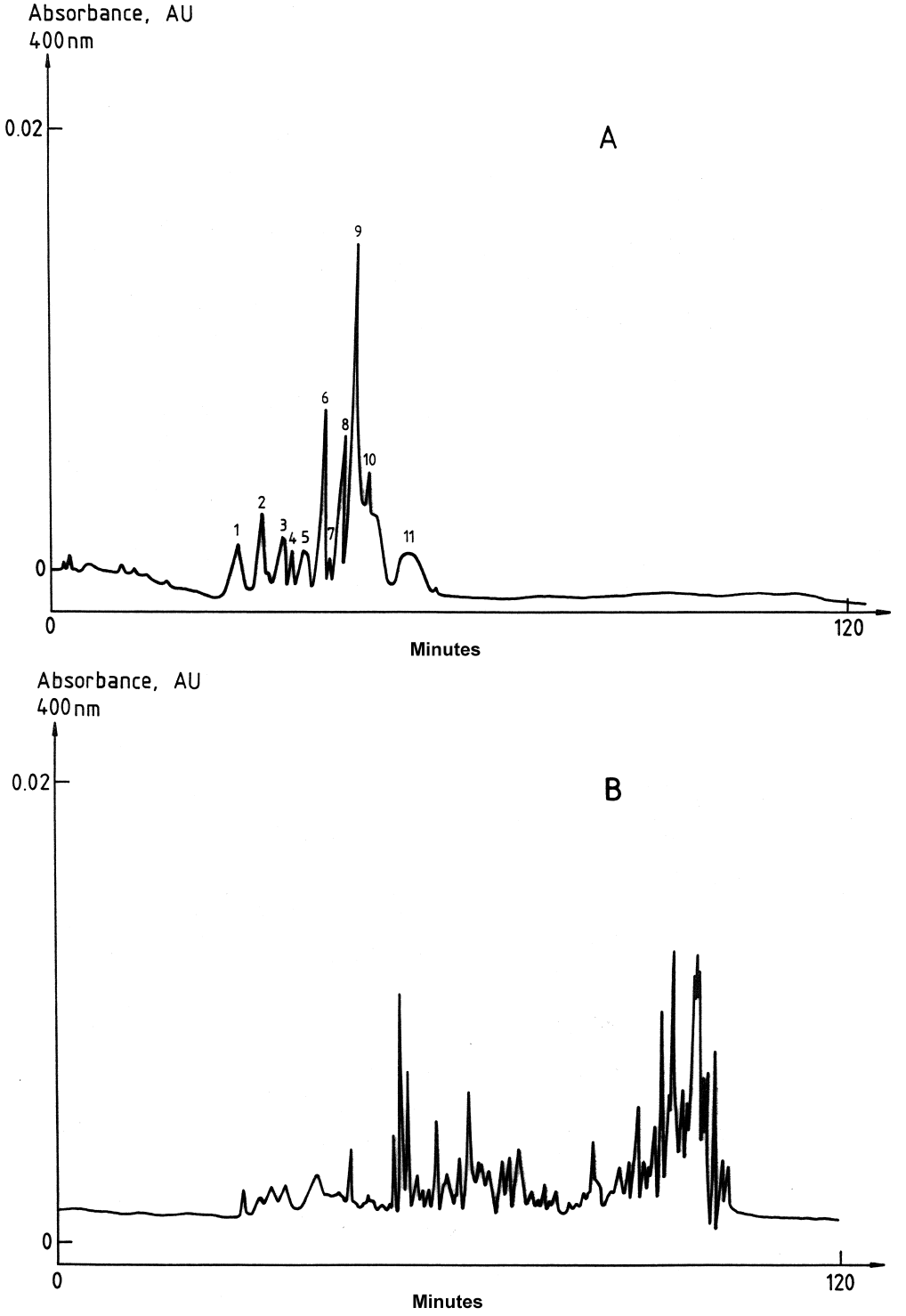

Fig. 3. RP-HPLC chromatogram of colour pigments of *Trichoderma harzianum* (A) and those of paprika (*Capsicum annuum*) powder (B).

Table 3
Retention time of pigment fractions of *Trichoderma harzianum* separated with gradient elution in RP-HPLC and inter- and intra-day reproducibility

Peak No.	Retention time (min)	Reproducibility (RSD, %)	
		Intra-day	Inter-day
1	28.30	1.35	1.60
2	32.08	1.07	1.45
3	35.34	1.21	1.67
4	36.51	1.42	1.71
5	38.39	0.98	1.28
6	41.65	1.23	1.56
7	42.85	1.40	1.81
8	44.65	1.14	1.54
9	45.68	1.31	1.82
10	48.64	1.42	1.57
11	55.12	1.00	1.23

validation parameters of the HPLC–DAD method are compiled in Tables 3 and 4. The RSD values are considerably lower than the corresponding values in RP-TLC. This result indicates the higher separation efficiency, stability and reliability of the HPLC–DAD system. However, also in this instance the RSD values of peak areas are markedly higher than those generally obtained in HPLC. This discrepancy can be tentatively explained by the fact that the baseline separation of pigments was not achieved even in RP-HPLC in each instances which adversely influenced the interaction process. The data further proved that the results of multistep gradient elution in RP-TLC can be successfully used to the rational design of gradient elution program in RP-HPLC. It has to be emphasized that this finding is not the result of theoretical considerations and it is only valid for this set of experiments. It is possible that its generalization may lead to serious error in the design of elution gradients in other chromatographic systems.

Table 4
Relative peak areas (%) of pigment fractions of *Trichoderma harzianum* separated with gradient elution in RP-HPLC and inter- and intra-day reproducibility

Peak No.	Peak area	Reproducibility (RSD, %)	
		Intra-day	Inter-day
1	7.54	3.21	3.45
2	6.18	3.17	3.56
3	4.99	2.91	3.21
4	1.92	3.48	3.85
5	4.60	3.25	3.67
6	12.92	2.11	2.39
7	1.14	3.85	4.27
8	10.71	3.00	3.51
9	37.54	2.15	2.69
10	1.90	3.78	4.02
11	10.56	2.09	2.61

3.3. HPLC–MS

HPLC–MS of the main fractions suggested a molecular mass of 470–500 and the presence of OH and NH_2 groups in the molecule, however, it was not able to identify their exact structure. The results of TLC–FT-IR and HPLC–MS are somewhat contradictory, the presence of amino groups in the pigment fractions was not confirmed by FT-IR. On the basis of TLC–FT-IR, HPLC–DAD and HPLC–MS it can be assumed that the main pigment fractions are oxidation polymers of low molecular mass originating from monomer molecules containing polar substructures and double bonds in the alkyl chain which are liable to oxidation during the aerobic fermentation process.

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