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FORMATION OF POLYMERIC PIGMENTS IN THE PRESENCE OF BACTERIA AND COMPARISON WITH CHEMICAL OXIDATIVE COUPLING—II. CATABOLISM OF TYROSINE AND HYDROXYPHENYLACETIC ACID BY *ALCALIGENES EUTROPHUS* CH34 AND MUTANTS

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Abstract—The formation of polymeric pigments using bacteria was investigated to produce new polymers. Polymeric pigments are formed from mutant strains of *Alcaligenes eutrophus* which are not able to open the aromatic ring (tyu$^-$) but transform tyrosine into *p*-hydroxyphenylacetic acid. This compound is then hydroxylated into homogentisic acid which undergoes oxidative coupling into polymeric pigments. Another carbon source is required to allow bacterial growth. The rate of disappearance of the initial product (either tyrosine or *p*-hydroxyphenylacetic acid) and the rate of formation and disappearance of intermediates were studied by ultraviolet (UV) absorption spectroscopy and high-performance liquid chromatography (HPLC). Pigment formation was monitored by gel permeation chromatography (GPC). The pigment was isolated and characterized by UV and infrared (IR) absorption spectroscopy, nuclear magnetic resonance (NMR) and elemental analysis. It was shown to have a complex structure involving aromatic and aliphatic saturated and unsaturated structures. Nitrogen is incorporated by condensation with amino acids and other nitrogen-containing molecules excreted by the bacteria. For comparative purposes, chemical oxidative coupling of homogentisic acid in the absence of bacteria was performed at pH 8.5 and 11.5 in the presence of oxygen. A badly defined but different mixed aliphatic–aromatic structure was isolated. The role of the bacteria in the different steps of transformation of the initial product is discussed. Copyright © 1996 Elsevier Science Ltd

1. INTRODUCTION

The use of micro-organisms for the production of polymers has been proposed many times in the last few years. The production of poly-β-hydroxybutyrate by *Alcaligenes* has been developed on an industrial scale [1]. Recently, 5,6-cis-dihydroxy-cyclohexa-1,3-diene (I) has been produced by oxidation of benzene by strains of *Pseudomonas putida* obtained by genetic manipulation in order to lock the enzyme responsible for the aromatization of I. Compound I was then derivatized into its diacetate, polymerized with radical initiators and aromatized to poly-*p*-phenylene [2]. The use of enzymes as catalysts for the synthesis of polyphenols has been proposed recently [3].

The polymeric pigments synthesized in the presence of syringaldehyde as carbon source by some strains of *P. putida* have been isolated in our laboratory and their chemical structure characterized in relation to the mechanism of bioassimilation of lignin model compounds. Pigment formation was shown to involve oxidative coupling of *o*-diphenol and *p*-diphenol [4]. Pigment formation by micro-organisms has been widely studied in the past [5]. Recent interest has been brought to melanin production by different strains of *Rhizobium* [6–8].

The present work has been initiated by the observation that *tyu*$^-$ mutants of *alcaligenes* do not grow when tyrosine is the lone carbon source, whereas they grow and produce a dark pigment in the presence of tyrosine and any other carbon source. The formation of dark pigments called pyomelanin in bacterial cultures containing L-tyrosine or phenylalanine has been reported previously [9, 10]. It is associated with a catabolic defect: homogentisic acid which is normally metabolized, accumulates in the medium and disappears when the pigment is formed. Pyomelanin is different from dopamelanin. Dopamelanin is produced by the oxidation of L-tyrosine, first into hydroxyphenylalanine (DOPA) and then in dopaquinone in the presence of tyrosinase; after cyclization and decarboxylation of dopaquinone into dihydroxyindole, condensation into dopamelanin of known structure occurs [5, 11]. The purpose of the present research is to elucidate the mechanism of pigment formation by *Alcaligenes eutrophus* in the presence of L-tyrosine or one of its metabolic products and to investigate pigment structure as a possible route to produce new polymers with interesting properties.

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The degradation pathways of tyrosine by micro-organisms are summarized in Scheme 1 [12]. Tyrosine is prepared for ring opening (steps e and g) by successive oxidation, hydroxylation and rearrangement (steps a–d). The products of ring cleavage then enter the usual metabolic cycles. Gram-positive micro-organisms generally follow steps a, b, c, g, h [13], while Gram-negative micro-organisms (*Pseudomonas aeruginosa*, *A. eutrophus*) degrade it according to a, b, d, e, f [14]. A soil yeast (*Trichosporon cutaneum*) degrades tyrosine into protocatechuic acid by a completely different mechanism [15] and finally opens the aromatic cycle.

2. EXPERIMENTAL PROCEDURES

2.1. *Micro-organisms*

Alcaligenes eutrophus CH34 was isolated from a decantation reservoir of a zinc factory with a high content of heavy metals. It is a chemolithotroph and displays resistance to heavy metals owing to the presence of two plasmids: pMOL28 (163 kb) (resistance to Ni, Co, Cr, Hg and Tl), and pMOL30 (238 kb) (resistance to Zn, Cd, Co, Cu, Pb, Hg and Tl).

Alcaligenes eutrophus CH34 can utilize a great variety of carbon sources (gluconate, succinate, malate, azelate, lactate, pyruvate, acetate, benzoate, *p*-hydroxybenzoate, phenol, histidine, threonine, tryptophane, proline, glycine, etc.) but not the sugars (glucose, arabinose, lactose, maltose). Optimal growth is obtained at 29°C. At 37°C a great probability of death and of spontaneous mutations among the survivors is observed [16]. Mutants Aut^-, Nit^-, Lys^- and tyu^- (unable to open the aromatic ring of tyrosine) have been isolated and identified.

Strain AE7 is a spontaneous mutant *tyuB7* obtained by temperature-induced mutagenesis. It is not able to open the ring of HGA and produces pigment. Such pigment is particularly conspicuous in broth media.

Strain AE150 (mutant *tyuA*150) is also a spontaneous mutant of *A. eutrophus* CH34, but it does not produce pigment.

2.2. *Culture medium*

Bacteria were grown on Luria agar and then transferred to minimal medium [KH_2PO_4 (0.5 g l^{-1}), $K_2HPO_4 \cdot 3H_2O$ (1.0 g l^{-1}), NH_4NO_3 (1.0 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.2 g l^{-1}), $FeSO_4 \cdot 7H_2O$ (0.01 g l^{-1})] provided with carbon source: lactate (2.5 g l^{-1} unless otherwise stated) and (or) aromatic compound (0.5 g l^{-1} unless otherwise stated). The pH was adjusted to 7; it increased to 8.5 at the end of the incubation period. To prepare solid medium, 15 g l^{-1} agar was added to the liquid medium. The incubation temperature was 30°C.

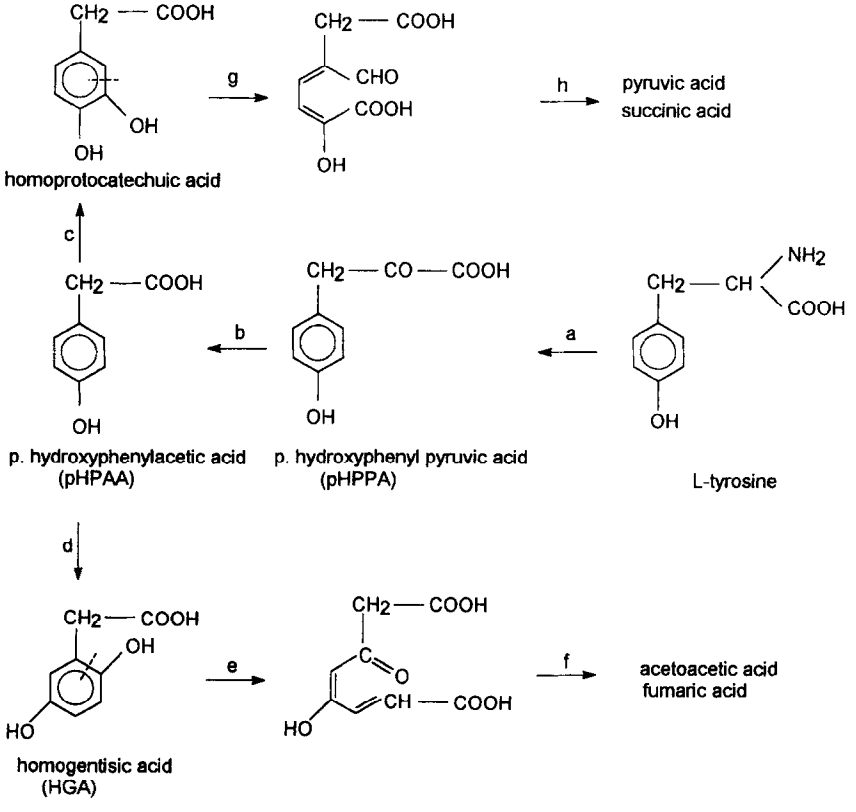
The aromatic compounds were Aldrich products: L-tyrosine, *p*-hydroxyphenylpyruvic acid (pHPPA), *p*-hydroxyphenylacetic acid (pHPAA), and homogentisic acid (HGA).

2.3. *Chemical oxidative coupling*

The reaction was performed at room temperature, at pH 11.5 and at pH 8.5. At pH 11.5, homogentisic acid (2 g l^{-1}) was stirred for different time intervals in NaOH solutions. The pH was readjusted each day. The reaction was stopped by adding HCl to a final pH of 2. At pH 8.5, HGA was stirred in the KH_2PO_4/K_2HPO_4 buffer at 30°C.

2.4. *Ultraviolet (UV) spectral analyses*

UV spectra of culture supernatants were recorded as a function of time with a Perkin-Elmer Lambda 3A spectrophotometer.



Scheme 1. Degradation pathways of tyrosine by micro-organisms.

Table 1. Growth (G) on agar medium of *A. eutrophus* (minimum medium + aromatic compound) and pigment formation (P) (minimum medium + lactate + aromatic compound)

Strain	CH34		AE7		AE150	
Aromatic compound	G	P	G	P	G	P
Tyrosine	+	−	−	+	−	−
pOH phenylpyruvic acid	ND	−	ND	+	ND	−
pOH phenylacetic acid	+	−	−	+	+	−
Homogentisic acid	+	−	−	+	+	−
Protocatechuic acid	+	−	+	−	+	−
Vanillic acid	+	−	+	−	+	−
Syringaldehyde	−	+	−	+	−	ND

ND = not determined.

2.5. *Infrared (IR) spectroscopy*

FTIR spectra of the acidified pigment (concentrated, dialysed and isolated by lyophilization) were recorded with a Bruker IFS45 spectrophotometer in KBr discs.

2.6. *Liquid chromatography*

After sterile filtration, aliquots were analysed using a Perkin-Elmer chromatograph, model 601, equipped with a high-performance Waters Novapak C18 column. The mobile phase was a ternary mixture of water/acetonitrile/acetic acid in a ratio 94/5/1, with a flow rate of 0.5 ml min^{-1}. Detection was made at 275 nm with a Perkin-Elmer LC55 UV spectrophotometer. Calibration was performed with Aldrich standards.

2.7. *Gel permeation chromatography (GPC)*

The column was a Waters Ultrahydrogel 250 for high-performance GPC of water-soluble polymers. The mobile phase was a 20% acetonitrile/80% phosphate buffer at pH 7 (KH_2PO_4 25 mM/K_2HPO_4 25 mM) with a flow rate of 0.8 ml min^{-1}. Detection was made at 265 nm with a UV Perkin-Elmer LC55 spectrophotometer. Calibration was performed with PEG and PEO standards, and an LDC refractive index detector.

3. RESULTS

3.1. *Growth and pigment formation on agar medium*

Growth and pigment formation for *A. eutrophus* CH34 and its two tyu$^-$ mutants were followed on agar medium supplemented with various aromatic compounds (Table 1).

Pigment formation is associated with a defect in the metabolism of aromatic compounds which cannot be used as sole carbon source by *tyuB* mutants of *Alcaligenes*. The wild-type strain *A. eutrophus* CH34 is able to open the aromatic ring and to metabolize all compounds with the exception of syringaldehyde. As a consequence, pigment is produced only in the presence of the latter compound when another carbon source (lactate) or broth is present to allow growth. The metabolism of four-substituted aromatic compounds such as syringaldehyde has been discussed previously [4]. Micro-organisms which are not able to open the ring successively oxidize it into syringic acid and demethylate this last compound into 3-*O*-methylgallic acid when lactate or vanillin is present to support growth. 3-*O*-methylgallic acid is then transformed into a complex nitrogen containing pigment by different chemical reactions involving oxidative coupling, condensation with nitrogen-containing molecules and ring opening.

Strain AE7 (*tyuB*) grows neither on tyrosine nor on its degradation products (pHPAA, HGA) and behaves as CH34 in the presence of the other aromatics. As in the preceding case, pigment is formed in the presence of products which cannot be metabolized by the bacteria, when another carbon source is present to allow growth.

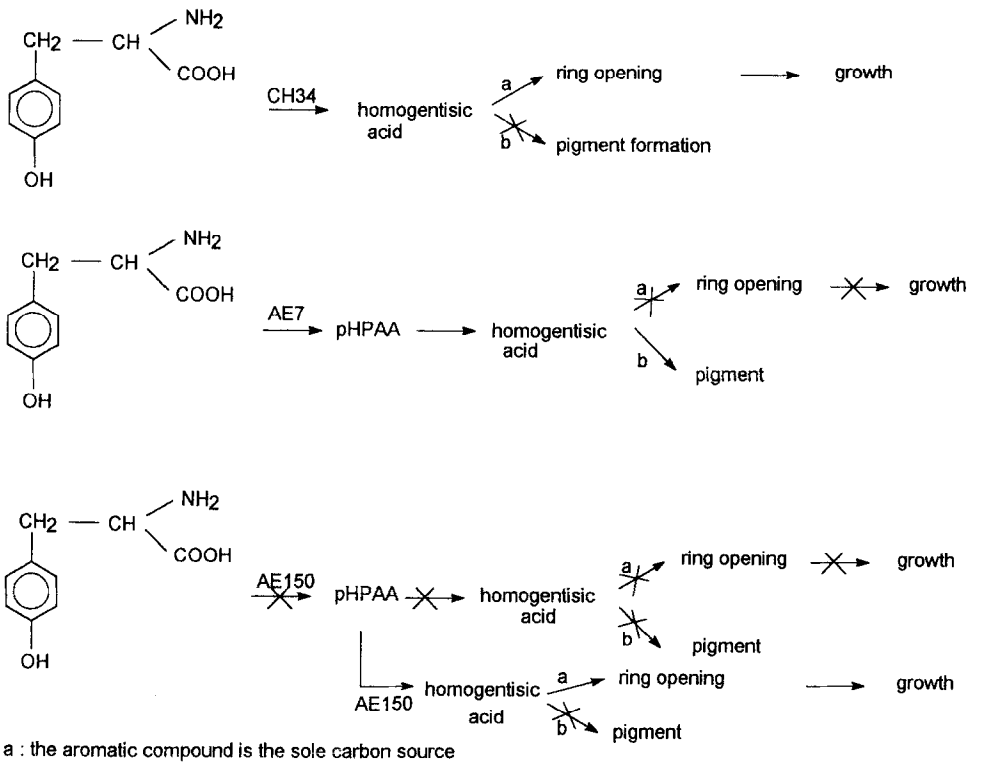
Mutant AE150 (*tyuA*) does not produce pigment in the presence of tyrosine and lactate although it cannot metabolize tyrosine. The absence of pigment formation from tyrosine in the presence of lactate and the absence of growth of AE150 on pure tyrosine indicate, according to Scheme 1, that the degradation sequence is interrupted either at step a or at step b. Growth is observed when pHPAA is the sole carbon source, demonstrating that steps d, e and f occur in the presence of AE150. For bacteria grown in the presence of syringic acid and an easily assimilable carbon source [4], pigment formation by oxidative coupling in the presence of O_2 requires the presence of *o*-diphenol or *p*-diphenol. In the present case and according to Scheme 1, it could thus only form from homogentisic acid in cases where ring opening does not occur.

The pathways to ring opening and pigment formation for the different strains tested are summarized in Scheme 2.

3.2. *Metabolism of tyrosine and of pHPAA and formation of pigment in liquid culture of* A. eutrophus *AE7*

The growth of bacteria, disappearance of substrates, formation and disappearance of intermediates and the formation of pigment (P_{bact}) were monitored by different methods [high-performance liquid chromatography (HPLC), UV absorption spectroscopy and GPC] as a function of the incubation time, for different initial concentrations of aromatic substrate and lactate. The pigment was then isolated and characterized by C, H, O, N elemental analysis, IR spectroscopy, GPC and nuclear magnetic resonance (NMR).

3.2.1. *Comparative degradation of tyrosine and pHPAA (initial concentration* 0.1 g l^{-1}). The concentrations of bacterial cells, substrate and intermediate products are given in Table 2 for tyrosine and in Table 3 for pHPAA. The degradation of tyrosine seems to proceed according to steps a, b, d, e, f of Scheme 1. Steps a, b and d are identified by the formation of pHPAA and HGA. pHPAA is a rapidly metabolized intermediate in tyrosine metabolism since it is detected only transiently. Lactate allows excellent bacterial growth in both cases. Formation of a brown pigment is revealed by the brown colour of the suspension and is already observed after 24 hr incubation. Since tyrosine and pHPAA follow the same degradation scheme, the second aromatic



Scheme 2. Formation of polymeric pigments from L-tyrosine in the presence of *Alcaligenes eutrophus*.

compound was preferred for this study because of its better solubility in the culture medium.

3.2.2. *Metabolism of pHPAA as a function of incubation time and initial pHPAA concentration at constant lactate concentration* (2.5 g l^{-1}). *Alcaligenes eutrophus* AE7 was grown using lactate as a carbon source in the presence of different concentrations of pHPAA (Table 3). Production and disappearance of HGA were followed by HPLC. Bacterial growth was similar in all cases. HGA intermediate was observed in all cases. Only 40% of the initial pHPAA disappeared at the highest (2 g l^{-1}) initial concentration but total degradation was observed at 0.1 and 0.5 g l^{-1}. The results concerning pHPAA at an initial concentration of 0.5 g l^{-1} will now be considered in more detail. Figure 1 gives the concentration of pHPAA and HGA as a function of time. Figure 2(a–d) gives the corresponding changes in the UV absorption spectra. The initial spectra at pH 7 (curve a) corresponds to *p*-hydroxyphenylacetate ($\lambda_{max} = 275$ nm) with probably a small amount of the corresponding phenolate ($\lambda_{max} = 295$ nm) with respective ϵ values of 1500 and 2400 litre $mole^{-1}$ cm^{-1}. Homogentisate at pH 7 absorbs at 290 nm with an ϵ value of about 3000 litre $mole^{-1}$ cm^{-1}. The absorption of this intermediate is thus superimposed on that of the phenolate (curve b). Pigment formation was identified by the brown colour which appeared after 24 hr and the continuous absorption in the long wavelength range (Fig. 2c, d). Bacterial growth was very good (3×10^9 ml^{-1} at the stationary phase). The GPC diagram obtained after 24 hr is given in Fig. 3. Residual pHPAA (retention time $t_r = 12.8$ min) and small quantity of HGA ($t_r = 15.5$ min) were observed. The peak at 10.2 min is due to pigment. Its molecular weight has been evaluated to about 3000 using polyethylene-glycols and polyethylene oxide as calibration standards and assuming that the pigments have the same molecular weight (MW) as the standards for identical retention times. The other peaks have not been identified.

Table 2. Metabolism of L-tyrosine by *A. eutrophus* AE7 (lactate: 2.5 g l^{-1})

Time (hr)	Initial L-tyrosine: 0.1 g l^{-1} Bacteria/ml ($\times 10^8$)	L-tyrosine (mg l^{-1})	pHPAA (mg l^{-1})	HGA (mg l^{-1})
0	1.3	83	0.0	8.2
3	2.0	68	0.0	17
6	4.8	43	3.7	32
9	8.2	3.2	0.0	50
12	10	3.6	0.0	46[a]
24	24	0.0	0.0	4.8[a]

[a]After 12 hr, a light brown colouration due to pigment was visually observed; after 24 hr, a dark brown colouration was observed.

3.2.3. *Metabolism of pHPAA at different lactate concentrations. Alcaligenes eutrophus* AE7 was incubated in the presence of pHPAA (2 g l^{-1}) using different initial concentrations of lactate (0.5 g l^{-1}, 1 g l^{-1} and 2.5 g l^{-1}) as a carbon source. The results corresponding to 2.5 g l^{-1} lactate are given in Table 3. The main difference between the cultures lay in number of bacteria at the stationary phase, which had the respective following values: 8×10^8, 2×10^9 and 3×10^9 ml^{-1}. pHPAA was only partly degraded in all cases. The amount of pHPAA degraded increased

Table 3. Metabolism of *p*-hydroxyphenylacetic acid by *A. eutrophus* AE7 (lactate: 2.5 g l^{-1})

Time (hr)	pHPAA: 0.1 g l^{-1}			pHPAA: 0.5 g l^{-1}			pHPAA: 2 g l^{-1}		
	bacteria/ml ($\times 10^8$)	pHPAA (mg l^{-1})	HGA (mg l^{-1})	bacteria/ml ($\times 10^8$)	pHPAA (mg l^{-1})	HGA (mg l^{-1})	bacteria/ml ($\times 10^8$)	pHPAA (mg l^{-1})	HGA (mg l^{-1})
0	1.2	88	5.0	1.5	480	11	1.8	1940	0
3	1.7	78	16	1.9	470	23	1.5	ND	ND
6	3.2	65	26	2.5	ND	ND	2.6	1920	37
9	8.2	55	ND	4.3	460	22	4.3	ND	ND
12	9.9	38	36[a]	5.4	430	40[a]	6.0	1880	59[a]
24	30	6.1	0.0[a]	26	300	75[a]	25	1570	270[a]
30	30	ND	ND[a]	30	190	26[a]	30	1180	82[a]
Final 48 hr	30	ND	ND[a]	30	45	0[a]	30	1150	0[a]

ND = not determined.
[a]After 12 hr, a light brown colouration due to pigment was visually observed; after 24 hr and more, a dark brown colouration was observed.

with the initial lactate concentration; the values corresponding to 0.5, 1 and 2.5 g l^{-1} lactate are 0.25, 0.6 and 0.8 g l^{-1} of degraded pHPAA, respectively.

These results, joined to those of the preceding paragraph, demonstrate that the amount of pHPAA oxidized and cometabolized into pigments is limited by the quantity of the carbon source. Furthermore, even at high initial lactate content (2.5 g l^{-1}), a maximum amount of 0.8 g l^{-1} pHPAA is transformed into pigments.

3.2.4. *Pigment isolation and characterization.* The pigment was isolated from a macroculture containing 0.5 g l^{-1} pHPAA and 2.5 g l^{-1} lactate. After the disappearance of more than 90% of the initial pHPAA, and acidification to pH 2, the bacteria were separated by filtration on a 0.2 μm Millipore filter. The solution was then partly evaporated. A first fraction of the pigment was separated as a suspension and isolated by centrifugation. This fraction corresponds to 85% of total isolated pigment. A second fraction was obtained by extraction of the residual coloured solution with ethyl acetate. After dialysis on Spectra/Por 3 membrane to eliminate salts and low molecular weight oligomers, followed by lyophilization, these two fractions represented about 30 wt% of the initial pHPAA.

The elemental analysis of fraction I gave: C (51.06%); H (3.35%); O (34.51%); N (4.11%). The incorporation of nitrogen probably resulted, as in humic substances [17], and syringaldehyde pigment [4], from condensation reactions of the aromatic compounds with amino acids excreted by bacteria.

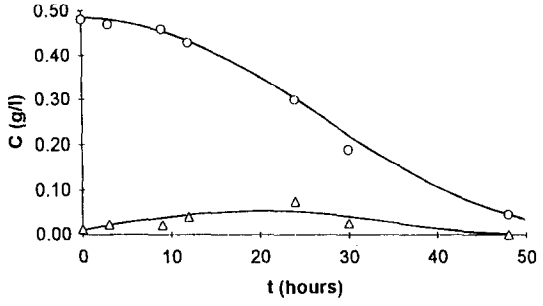


Fig. 1. Concentration in pHPAA (○) and HGA (△) as a function of time, for *A. eutrophus* AE7 growing on pHPAA 0.5 g l^{-1}.

The IR spectra of the first and second fraction are given in Figs 4 and 5. The following absorptions have been identified:

(i) at 3320 cm^{-1}, associated OH from alcohols and acids;
(ii) at 2920 cm^{-1}, aliphatic C–H;
(iii) at 1715 cm^{-1}, carbonyl stretching from saturated acids;
(iv) at 1610 cm^{-1}, a broad absorption which can include various carbonyl stretching from unsaturated ketones and acids, quinones, amino acids and amides.

The first and second fractions show similar characteristic IR absorptions with an important difference in relative optical density of the bonds situated at 1715 and 1610 cm^{-1}.

The UV spectrum of the pigment in the culture medium (Fig. 2d) is identical to that of isolated fraction I and does not exhibit any well-defined absorption; this continuous spectrum is characteristic of long sequences of conjugated double bonds. It is quite different from the spectrum obtained for the pigment resulting from the condensation of syringaldehyde in the presence of *Pseudomonas* [4].

The NMR spectrum of the first fraction (not shown) is badly resolved but reveals the presence of aromatic protons, methylene protons linked to an aromatic cycle, unsaturated and saturated aliphatic protons resulting probably from ring opening and from amino acids.

3.3. *Oxidative coupling of HGA in the absence of bacteria at pH 11.5 and 8.5*

Since chemical oxidative coupling was initially suspected to be involved in pigment formation, this reaction was investigated separately, in the absence of bacteria, to compare the structure of the pigment formed in the presence and in the absence of microorganisms. pHPAA is not a possible substrate for oxidative coupling since two hydroxyls in ortho or para positions to each other are required. HGA, which results from hydroxylation and rearrangement of pHPAA by bacteria, was thus chosen as the starting product.

The GPC of the polycondensation chemical medium at pH 11.5 corresponding to an initial HGA concentration of 0.4 g l^{-1} is shown in Fig. 6. After 0.5 hr, various condensation products were detected. Residual HGA has a t_r of 15.5 min. After 2 hr, HGA had completely disappeared. After 24 hr (diagram not shown), the retention time of the chemically produced

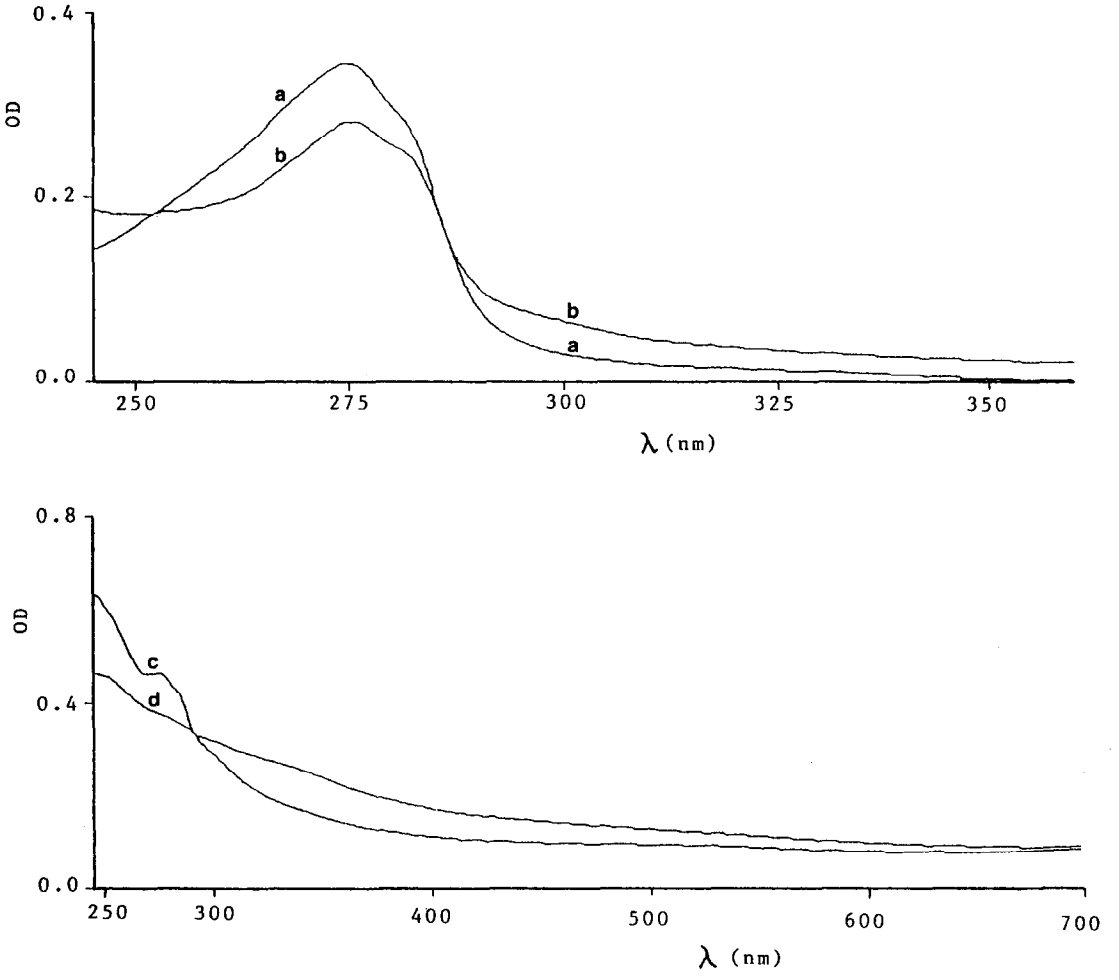


Fig. 2. UV spectra of the culture medium for *A. eutrophus* AE7 growing on pHPAA 0.5 g l^{-1} (dil. 4×) after (a) 0 hr, (b) 12 hr, (c) 24 hr, (d) 48 hr.

pigment ($P_{ch(11.5)}$) was 10.7 min. The corresponding molecular weight is 1700 if the same approximation as in the preceding section is used. The pigment $P_{ch(11.5)}$ was isolated from solutions initially containing 2 g l^{-1} HGA. The condensation reaction was stopped by acidification on resin up to pH 2, followed by dialysis and lyophilization. The spectral characteristics of the pigment are similar after 24 or 96 hr. The UV spectrum (not shown) presents a continuous absorption extending up to the visible and similar to that of P_{bact} given in Fig. 2d but, in addition, a well-defined absorption at 270 nm is now superimposed on this continuous absorption ($\epsilon = 4000$ litre $mole^{-1}$ cm^{-1} assuming that the condensation unit is HGA). The IR spectrum (Fig. 7) is similar to that of fraction II obtained in the presence of bacteria but the absorption at 1500 cm^{-1}, which probably results from aromatic groups, is absent. Aromatic protons are nevertheless detected by ^{1}H-NMR, together with unsaturated aliphatic protons resulting from ring opening and methylene protons from the side-chain.

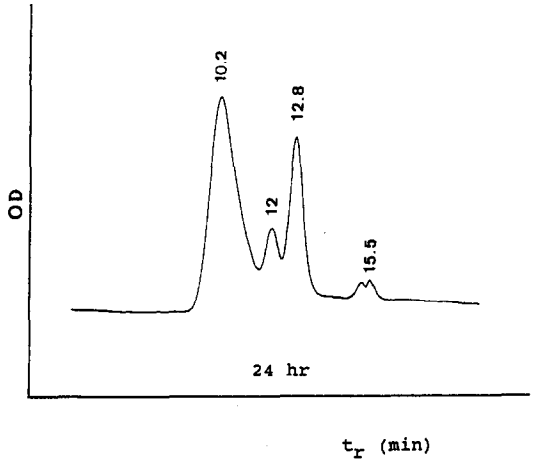


Fig. 3. GPC of the culture medium for *A. eutrophus* AE7 growing on pHPAA 0.5 g l^{-1} (dil. 2×) after 24 hr growth.

When the condensation was performed at pH 8.5, the rate of disappearance of HGA was much lower than at pH 11.5. Several condensation products were formed (Fig. 8). After 9 days, only one peak with a

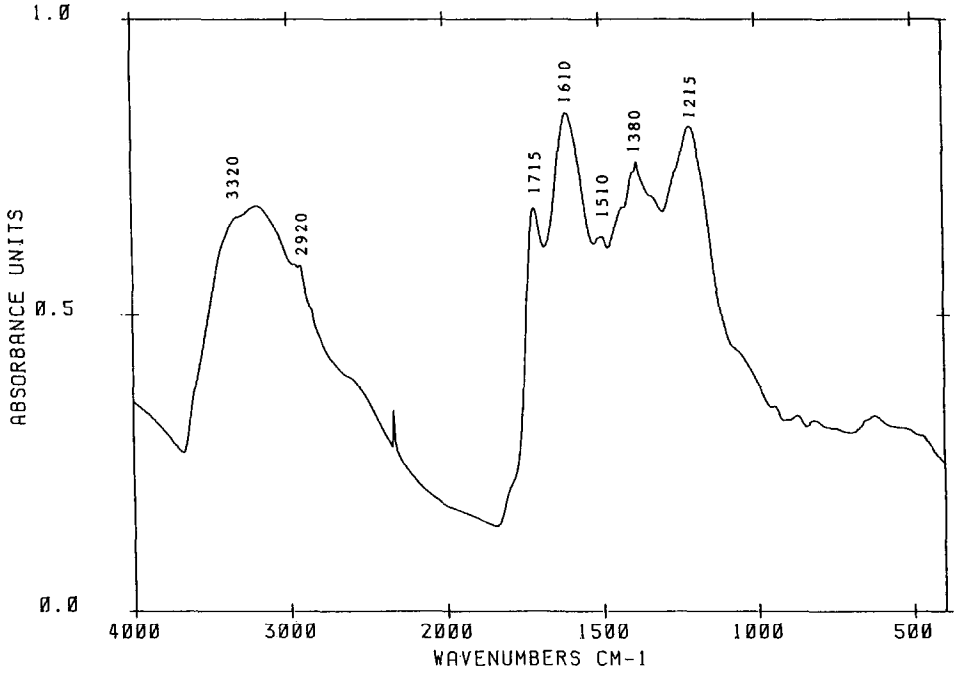


Fig. 4. FTIR spectrum of the bacterial pigment formed from pHPAA (fraction I).

retention time t_r of 10.7 min was observed. The disappearance of the initial aromatic compound was slower than for pHPAA (Fig. 3) in the presence of bacteria at the same pH. The pigment $P_{ch(8.5)}$ formed in this condition was isolated as previously. The elemental analysis gave: C (44.84%); H (3.15%); O (41.72%). The UV and IR spectra are similar to those of P_{bact} and $P_{ch(11.5)}$ but ϵ has been evaluated to 3000 litre mole^{-1} cm^{-1} in this case at 270 nm. Evidence for the presence of aromatic groups in the polymer is obtained from the ^{1}H-NMR spectra where aromatic and methylene protons linked to an aromatic ring are clearly discernible.

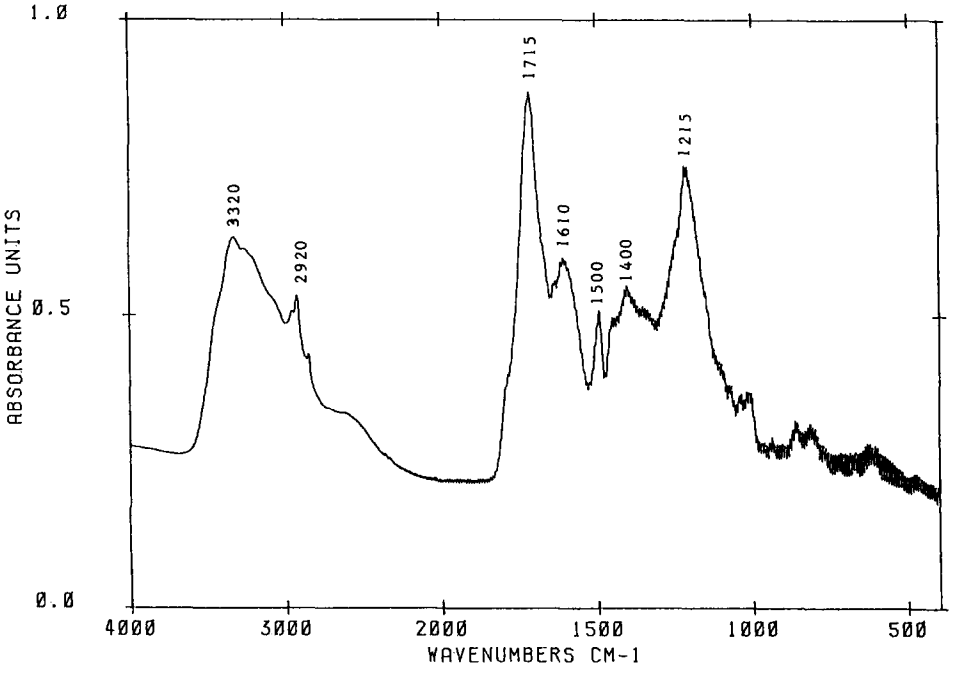


Fig. 5. FTIR spectrum of the bacterial pigment formed from pHPAA (fraction II).

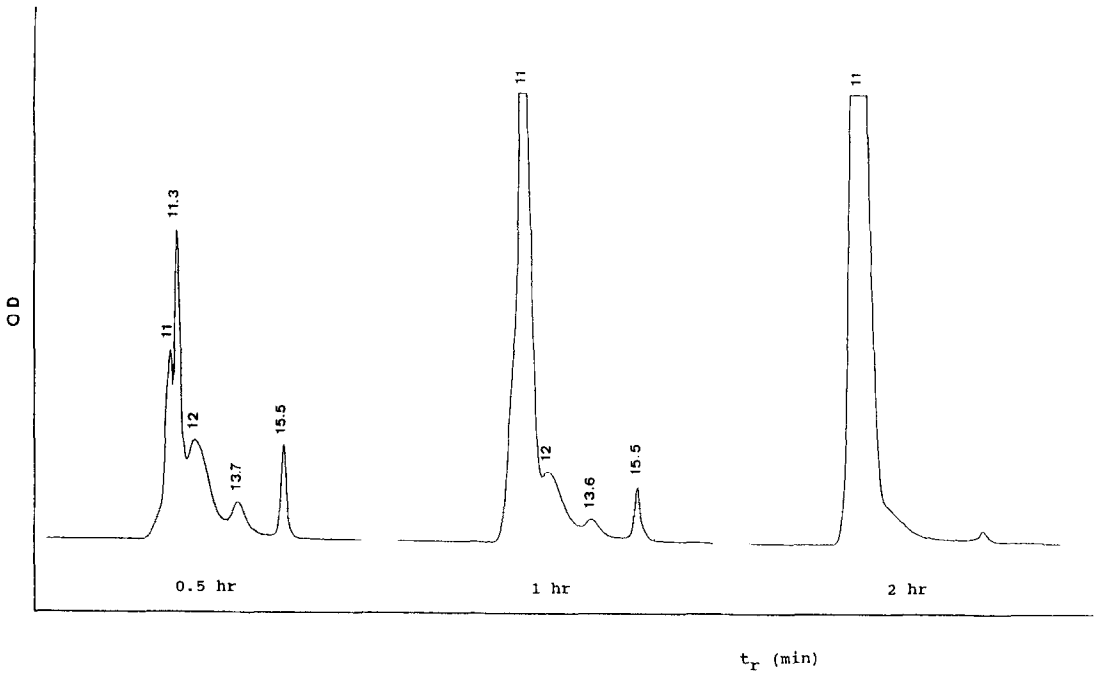


Fig. 6. GPC of the chemical condensation medium of HGA 0.4 g l^{-1} at pH 11.5, after 0.5 hr, 1 hr and 2 hr.

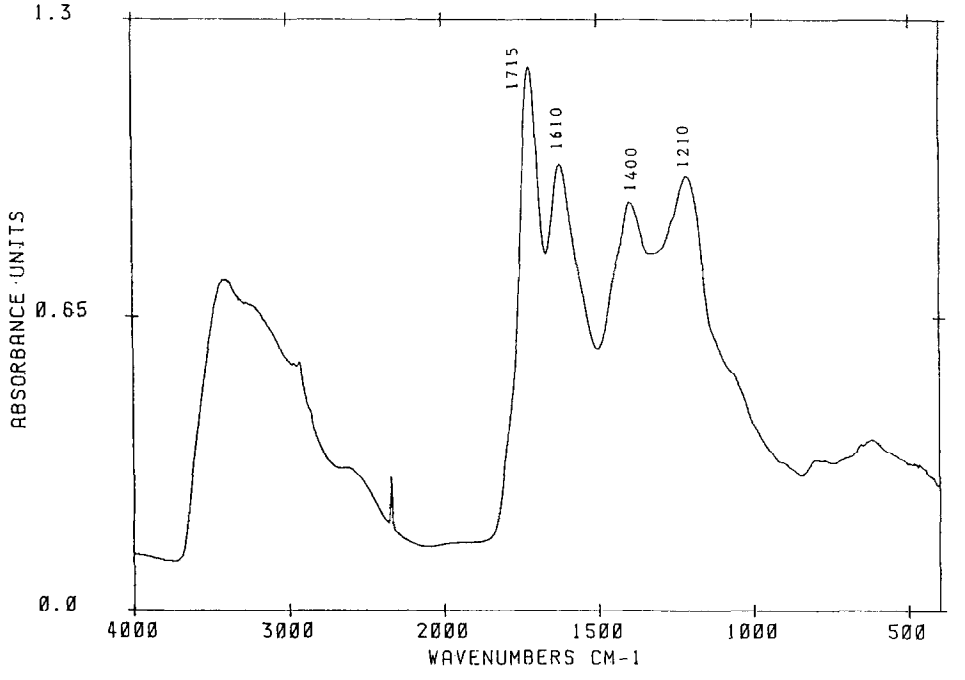


Fig. 7. FTIR spectrum of the chemical pigment formed after several days from HGA at pH 11.5.

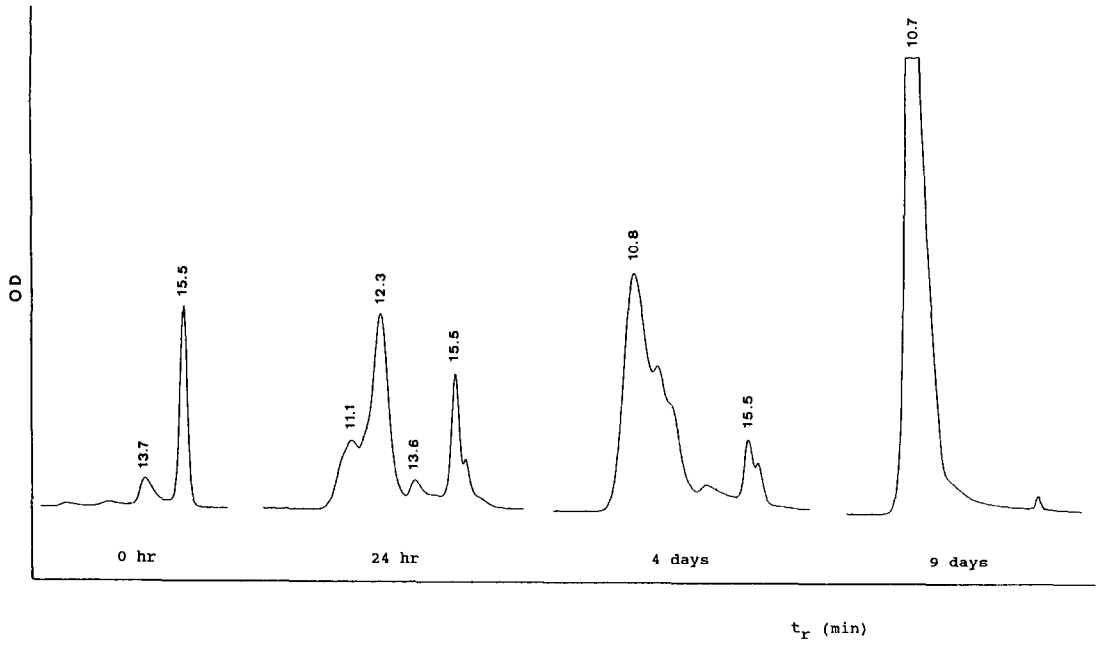


Fig. 8. GPC of the chemical condensation medium of HGA 0.4 g l^{-1} at pH 8.5, after 0 hr, 24 hr, 4 days and 9 days.

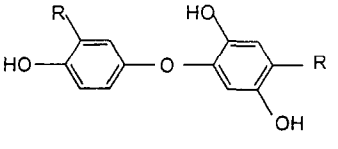
DISCUSSION

All the methods used to determine the structure of the pigments gave clear evidence that P_{bact}, $P_{ch(11.5)}$ and $P_{ch(8.5)}$ have badly defined structures and do not differ much in molecular weight. The same conclusion was drawn previously for the pigments obtained from syringic and 3-*O*-methylgallic acid [4]. P_{bact} contains nitrogen resulting from condensation with amino acids or proteins excreted by the bacteria. All three pigments probably have a mixed aromatic aliphatic structure with the proportion of aromatic groups decreasing in the order $P_{ch(8.5)} > P_{bact} > P_{ch(11.5)}$. Also, the proportion of carboxyl group is lower in P_{bact} (fraction I) than it is in $P_{ch(8.5)}$ or in $P_{ch(11.5)}$.

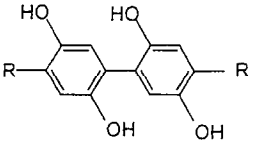
The formation of P_{bact} from either tyrosine or pHPAA involves the participation of the micro-organisms at different levels:

(i) preparation of the molecule to oxidative coupling by various successive oxidation and rearrangement steps to produce HGA according to Scheme 1.
(ii) synthesis of the amino acids or other nitrogen-containing molecules which are incorporated in the pigment.
(iii) probable participation in the condensation reaction. Indeed, pigment formation is more rapid from pHPAA in the presence of bacteria than it is at the same pH when HGA is the starting product.

Many possibilities exist for the oxidative coupling of HGA in alcaline medium. A few examples of these possibilities are given in Scheme 3. Structures III and II will not be very reactive owing to steric effect at the level of the unpaired electron. IV and V rearrange into:



VI

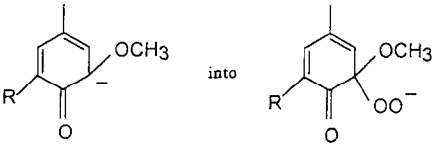


VII

which can be involved in other coupling reactions since two phenolic OH are still available.

Other possible reactions involve oxygen addition on intermediate radicals II, II′ and III.

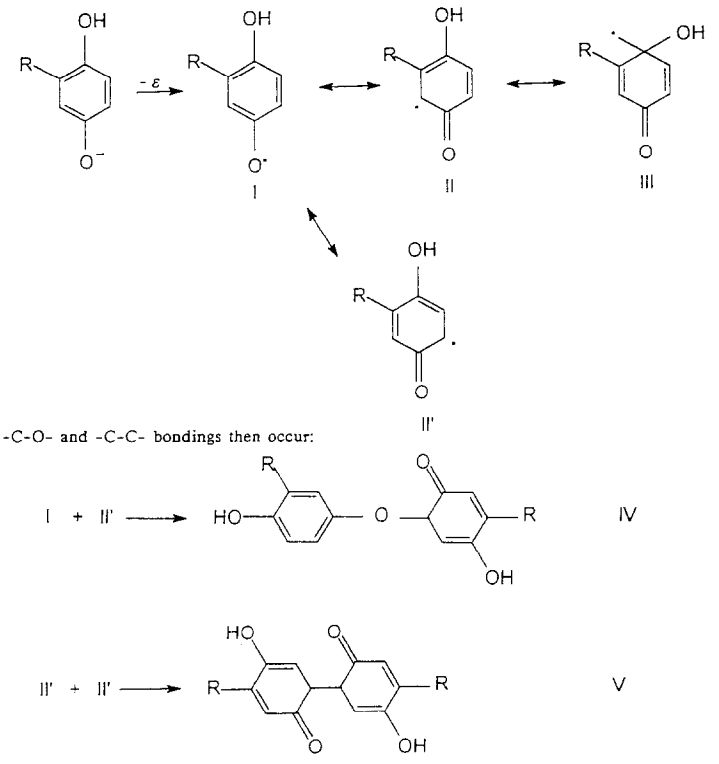
In a strongly alkaline medium, it is well known that ring opening of syringyl and gaïacyl structures occurs by oxygenation of carbanion



intermediate which undergoes ring opening via a dioxetane compound [18]. Such a reaction could also occur in the condensed phenolic structures of type IV to VII and justify the aliphatic unsaturated structures observed in $P_{ch(11.5)}$.

It is well known that oxidative coupling usually gives a complex mixture of products, generally dimers, oligomers and sometimes polymers [19, 20]. This complexity is due to several factors, including: coupling involves the phenoxy radical and its *o*- or *p*-position. Substitution of the aromatic ring thus plays an important role; free radicals but also ArO^+ cations have been proposed to be involved in coupling according to the strength of the oxidant and the pH of the medium [20].

Many natural polymers result from oxidative coupling; they include bacterial, fungal and mammalian pigments and also lignin. Most of them have a



Scheme 3. Examples of oxidative coupling steps for HGA in alcaline medium.

complex structure, the most thoroughly investigated being lignin. Only two cases of synthetic polymers with a well-defined structure have been reported among a large number of non-specific oligomers and polymers [3, 21].

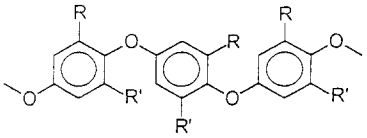
Our results, jointed to those of the literature, give an insight into the future of microbiological methods for the synthesis of polymers.

Condensation reactions involving esterification or formation of glycosidic linkages at well-defined sites of small molecules in the presence of micro-organisms are well known and will probably undergo an important development. One famous example is poly-β-hydroxybutyrate, an energy and carbon storage material for some micro-organisms. Copolymers and other polymers [poly(β-hydroxy-5-phenylvalerate), for example] were also synthesized recently in the presence of micro-organisms [1]. The biosynthesis of cellulose by bacteria is a trivial example of glycosidation.

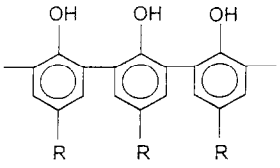
Condensation involving oxidative coupling is less favourable. In the presence of micro-organisms or enzymes, only badly defined structures have so far been reported. However, the use of enzymes in mixed aqueous organic solvent [3] or in micro-emulsions [22] opens new perspectives. The use of oxygen as an oxidant is particularly unfavourable since only orthodiphenols or paradiphenols are oxidized by O_2. Diphenols will inevitably result in branching. Well-defined linear polymers can be obtained in only two cases:

(i) selective C–O coupling in the para position of *o*-disubstituted monophenols (structure a), although this is not very favourable owing to steric effects;

(ii) selective C–C coupling in the ortho position of para-substituted monophenols (structure b):



Structure a



Structure b

Examples of these structures are, respectively, the poly-*p*-phenylphenols obtained by Akkara *et al.* [3, 22] in the presence of horseradisperoxidase, and the poly-*p*-phenylene oxides obtained by Hay [21] in the presence of metal oxides.

Much work needs to be done to understand how to select the oxidant, catalyst (eventually enzymes) and solvent to favour this specificity.

It must be noted here that several conducting polymers with a regular structure (polypyrroles, polythiophenes, polyanilines, etc.) have been obtained by electrochemical oxidative coupling [23].

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