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Protective Effect of Biomass Components Against Interfacial Inactivation of α-Pinene Oxide Lyase from *Pseudomonas rhodesiae* CIP 107491

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Summary

α-Pinene oxide lyase is able to catalyze the cleavage of both rings of α-pinene oxide to form *cis*-2-methyl-5-isopropylhexa-2,5-dienal (isonovalal) with no cofactor requirements. This bioconversion, when carried out under biphasic conditions (water/hexadecane) by *Pseudomonas rhodesiae* CIP 107491, allows the accumulation of a very high concentration of the product. Nevertheless, the reaction stopped due to the loss of enzyme activity, which has been demonstrated to be an irreversible phenomenon. The enzyme was purified by chromatographic methods to study the reasons of its instability. Bioconversion with pure enzyme showed that a protein precipitation occurred at the liquid-liquid interface, giving rise to a decrease in soluble protein and in residual enzymatic activity. The rate of decrease in soluble protein concentration was not related to the presence of the precursor, which indicated that the inactivation was interfacial, *i.e.* due to the direct contact of the enzyme with the hexadecane layer. This phenomenon was not present when crude enzymatic extract was used as a biocatalyst. Biomass components present in this extract thus had a protective effect against interfacial phenomena, demonstrating that α-pinene oxide lyase purification does not attract much practical interest for isonovalal production.

Key words: bioconversion, isonovalal, interfacial inactivation, *Pseudomonas rhodesiae* CIP 107491, protective effect

Introduction

Terpenes and terpenoids are the primary flavour and fragrance molecules found in the essential oils of higher plants. They are naturally branched chain C10 hydrocarbons formed from isoprene units and are widely distributed in nature (*1*). Terpenes are traditionally isolated from essential oils. Biotransformations of these compounds remain of great commercial interest for the food and perfume industry since one of the main advantages of using biotechnological methods for the production of flavour and fragrances is to avoid drawbacks associated with natural extraction processes. Comprehensive reviews by Krasnobajew (*2*) and more recently by Schrader and Berger (*3*) focused on many transformations of terpenoids for the flavour and fragrance industry.

Nevertheless, these compounds are generally both volatile and lipophilic, which poses several problems. Their low water solubility often leads to the presence of an excess substrate that gives an organic layer. They also often exhibit a rather low chemical stability when poured into an aqueous environment, and undergo spontaneous autooxidation processes. Their high volatility can additionally give rise to loss by air stripping, especially

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when the reaction is performed aerobically (*4,5*). Another difficulty, encountered when whole cells are used as biocatalyst, is their high toxicity. This feature is generally attributed to their high concentration in the cytoplasmic membrane, where hydrophobic compounds accumulate preferentially (*1,6*). As a consequence, only a few biotransformation processes in the area of terpenes have been proposed up to now at an industrial scale.

Among them, the bacterial synthesis of acyclic metabolites of α-pinene oxide has led to a patent (*7*) that claims the production of isonovalal (*cis*-2-methyl-5-isopropylhexa-2,5-dienal), a citrus, woody and spicy fragrance molecule (Fig. 1). Studies of α-pinene metabolism from *Pseudomonas fluorescens* NCIMB 11671 (*7–9*) or *Nocardia* sp. P18.3 (*10*) reported that the enzyme catalysing this reaction, α-pinene oxide lyase, is able to cleave both rings of the epoxide to form isonovalal with no cofactor requirement (Fig. 1).

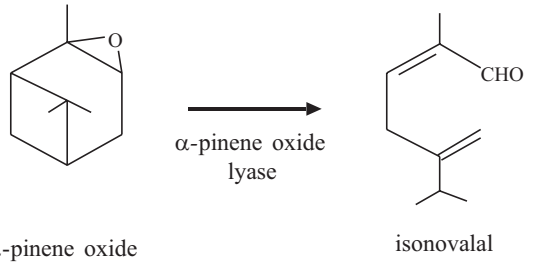

Fig. 1. Reaction giving isonovalal (*cis*-2-methyl-5-isopropylhexa-2,5-dienal) from α-pinene oxide

More recently, bioconversion of α-pinene oxide into isonovalal by a new strain, *Pseudomonas rhodesiae* CIP 107491, in a biphasic medium has been described (*11*). Three key parameters have been evidenced. The first is the need for a cell permeabilization by freezing and further contact with the thawed material by an organic solvent such as chloroform, toluene or diethyl ether. This operation allows both an enzyme release in the aqueous phase outside the cells and an improvement of transport properties of the substrate and product across the cell membrane, strongly increasing reaction rates. It also inactivates other enzymes of the precursor degradation pathway, enabling high yields to be achieved. The second one is that the enzyme α-pinene oxide lyase, which exhibits an irreversible inactivation, probably by a metabolite in the medium, presents a constant turnover, *i.e.* the total product synthesis is proportional to the biomass loading, and close to 16.4 g isonovalal/(L·g biomass). The third phenomenon is that the biphasic system used is not limited by phase transfers, a feature attributed to the spontaneous formation of an oil-in-water emulsion. It has then been possible to carry out a very efficient process allowing to recover 400 g/L of isonovalal with 25 g/L of biomass in 2.5-hour reaction, corresponding to an average reaction rate as high as 160 g/(L·h).

Then, in order to get a better understanding of the mechanisms involved in isonovalal formation by *P. rhodesiae*, and to increase the achievements of this process, the enzyme was purified. The purification method and the enzyme characterization have recently been published (*12*).

Results presented here aim at (*i*) determining if this additional step in the bioconversion process can, from a process point of view, be interesting for isonovalal production, and (*ii*) to gain further insights in the mechanism of the above-mentioned inactivation phenomenon.

Materials and Methods

Microorganism

Pseudomonas rhodesiae CIP 107491 (*11*) was maintained on nutrient agar (Difco) in Petri dishes and held at 30 °C. Successive replications were performed every 3 or 4 weeks. A stock material was also prepared using the Protect Bacterial Preserver System (Technical Service Consultants Ltd, Heywood, Lancashire, UK) which is made of porous ceramic beads immersed in a cryopreservative fluid stored at –75 °C (*13*).

Biocatalyst production

Two precultures were prepared in 500-mL Erlenmeyer flasks filled with 250 mL of *Pseudomonas* basal medium (*14*) and with 8 g/L of sodium lactate as carbon source (*15*). Flasks were inoculated with colonies picked out from an agar plate (see above). After 24-hour growth at 30 °C in a rotary shaker operated at 200 rpm, the absorbance at 600 nm (A_{600}) was close to 3 (biomass concentration 1.3 g/L).

Precultures were used to inoculate an aerated, stirred bioreactor (Biostat MD, B. Braun, Melsungen, Germany) of a 5-litre working volume. The medium was made of 4.3 L of *Pseudomonas* basal medium where sodium lactate is replaced by glucose (5.6 g/L) and 200 mL of an organic solution made of 15 mL of α-pinene in hexadecane (62 g/L of α-pinene per litre of hexadecane). The stirring rate was maintained at 500 rpm, the temperature at 30 °C and aeration rate at 30 L/h (0.05 vvm). pH decrease was limited to 6.0 using 0.5 M NaOH solution. The process was stopped after 9 to 12 h of cultivation.

The resulting broth was then centrifuged for 10 min at 5000×*g* and ambient temperature. The obtained pelleted cells were resuspended in an appropriate volume of KH_2PO_4/Na_2HPO_4 buffer (20 mmol/L, pH=7.1) to yield a biomass concentration close to 7 g/L (the relationship between biomass and absorbance was established as $X=0.447 \cdot A_{600}$). This concentrated suspension was stored frozen at –20 °C.

Purification of α-pinene oxide lyase

Protocol for α-pinene oxide lyase purification from *Pseudomonas rhodesiae* CIP 107491 has recently been described (*12*).

Biotransformation media

Reaction medium was a two-phase system comprising an organic phase made of an organic solution of α-pinene oxide in hexadecane. Initial substrate concentration was 20 g of α-pinene oxide per litre of hexadecane. According to the experiments, the aqueous phase

was composed of different fractions: (*i*) permeabilized biomass: cell suspension in phosphate buffer was thawed and poured in an Erlenmeyer flask. Before reaction, diethyl ether was added to the aqueous suspension (5 % by volume) for 1 h at 30 °C with the flask being placed in a rotary shaker operated at 200 rpm; (*ii*) biomass supernatant: fraction made of permeabilized biomass was centrifuged (10 min, 10 000×*g*) and filtered using 0.22-μm filter in order to remove cell debris and residual viable cells; (*iii*) crude extract: thawed biomass disrupted by sonication (Ikasonic U50 H) at a maximal energy of 125 W/cm. Three cycles of 2 min were performed for each sample, maintaining the tubes on ice, and with 5-minute cooling intervals. Suspensions were centrifuged at 20 000x*g* for 45 min at 20 °C and then filtered using 0.22-μm filter; (*iv*) purified enzyme: solution of purified enzyme in KH_2PO_4/Na_2HPO_4 buffer (20 mmol/L, pH=7.1).

The phase volume ratio was 2:5 (aqueous/organic phase), and the reaction temperature was 30 °C. Bioconversions were carried out in 100-mL Erlenmeyer flasks placed in a rotary shaker (200 rpm).

Biotransformation analysis

Organic phase was directly analysed by gas chromatography by injecting 1 μL of the sample into the apparatus. The chromatograph (HP 6890, Agilent Technologies, Palo Alto, CA, USA) was fitted with an apolar SPB-5 (Supelco Inc., Bellefonte, PA, USA) capillary column (30 m×0.32 mm i.d., film thickness 0.25 μm) and a flame ionisation detector. The carrier gas was nitrogen, and the oven temperature was kept at 80 °C for 5 min, then raised to 200 °C at an interval of 20 °C/min. Both the injector and detector temperatures were 250 °C; the split ratio for the injection was 1:5. Concentrations were calculated by assuming that all compounds had the same response factor as α-pinene oxide. Internal standard was heptadecane (1 g per litre of hexadecane).

Determination of protein content

Protein content of the samples was determined at each step using three different methods: Lowry method modified by Peterson (*16*), Coomassie blue method (*17*) and bicinchoninic acid method (*18*). Crystalline bovine serum albumin (BSA) was used as standard.

Zymography

A volume of 10 μL of samples (80 μL of protein solutions with 20 μL of bromophenol blue) was applied to polyacrylamide gel (12 % of acrylamide:N,N'-methylenebisacrylamide 37.5:1, 1 mm thickness, 8×6 cm, buffer: Tris/HCl, pH=8.9). The migration was carried out in vertical mode in a Bio-Rad Protean II apparatus (Bio-Rad) at 150 V with Tris/glycine migration buffer (pH=8.3) for 2 h until bromophenol blue (tracking dye) was 5 mm from the bottom of the gel. Gel was then immersed in an emulsion containing 20 mM α-pinene oxide in 20 mM phosphate buffer (pH=7.1). After 30 min of incubation, the position of lyase band was visualized by the presence of a white precipitate. Known quantities of purified α-pinene oxide lyase were used as standards. Unknown quantity bands were quantified using Quantity One Bio-Rad software (version 4) in 'trace density' mode.

Results and Discussion

Estimation of α-pinene oxide lyase content in different fractions

The purification process allowed the determination of the lyase content in crude extract based on bioconversion activity. Crude extract contained 200 mg of proteins in 50 mL of the solution, with an activity of 45 μmol/(min·mg), whereas after purification on DEAE-Sephacel column, the resulting sample consisted of about 40 mg of proteins with a 5-fold higher activity (120 μmol/(min·mg)). After purification on Superdex 75 column, around 15 mg of proteins were recovered, with an activity of 600 μmol/(min·mg). These data allowed to conclude that a 13-fold purification was obtained, indicating that α-pinene oxide lyase accounted for about 7.5 % of the proteins in the crude extract (*12*).

In order to confirm this results, a zymogram method was developed. Zymography is a routine electrophoretic technique to identify proteolytic activities in polyacrylamide gels under nondenaturing conditions. This method was adapted to detect α-pinene oxide lyase. Indeed, immersion of gels in a suspension containing 20 mM α-pinene oxide in phosphate buffer (pH=7.1) allowed to visualize the lyase band due to the presence of a white precipitate of the insoluble reaction product, *cis*-2-methyl-5-isopropylhexa-2,5-dienal (isonovalal). Lyase concentration could be correlated to the trace intensity of the precipitate. Calibration curve was established with purified enzyme thanks to Quantity One software (Bio-Rad) (Fig. 2). This method gave the same results as previously reported, *i.e.* the lyase content in the crude extract proteins was also close to 7.5 %.

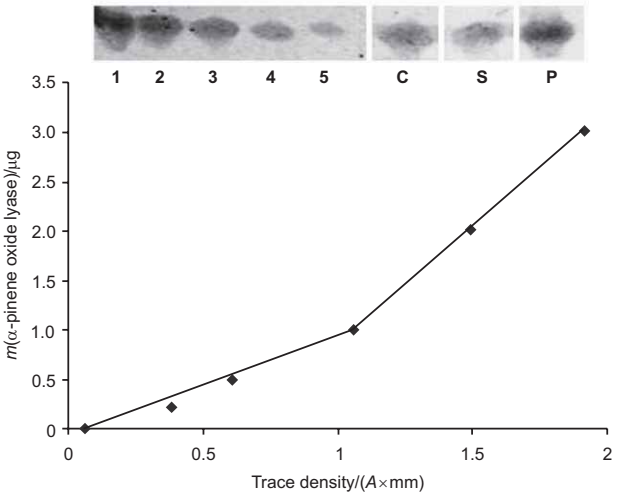

Fig. 2. Calibration curve obtained from zymogram technique. Lanes 1 to 5: zymogram obtained with different known quantities (3.02 to 0.22 μg) of pure α-pinene oxide lyase, C: zymogram of crude extract, S: zymogram of biomass supernatant, P: zymogram of permeabilized biomass

Then, zymogram method was applied to determine lyase content in other fractions like permeabilized biomass classically used for bioconversion or biomass supernatant. Results in Table 1 indicate that lyase content

Table 1. Determination of α-pinene oxide lyase content of several fractions by zymography

Fractions	*m*(lyase)	*γ*(lyase)	*γ*(total protein)	*w*(lyase)
	μg	g/L	g/L	%
Crude extract	2.15	0.27	3.61	**7.5**
Biomass supernatant	1.23	0.15	1.37	**11.3**
Permeabilized biomass	2.23	0.28	4.29	**6.5**

in crude extract (7.5 % of proteins) was slightly higher than in permeabilized biomass (6.5 %). Indeed, total protein concentration was higher in permeabilized biomass than in crude extract due to the presence of membrane proteins (see Materials and Methods section). However, the lyase concentration is practically the same in both cases, indicating that enzyme extraction during crude extract preparation was efficient. A more surprising result was the content of lyase in biomass supernatant (11.3 %), which seemed to indicate that the permeabilization step allowed the release of mainly α-pinene oxide lyase out of the cells. Results in Fig. 3 further confirmed that zymogram was a reliable method to quantify α-pinene oxide lyase content of a sample since for the same estimated quantity of lyase, rate of bioconversion and maximal isonovalal production are close for all fractions (crude extract, permeabilized biomass, biomass supernatant).

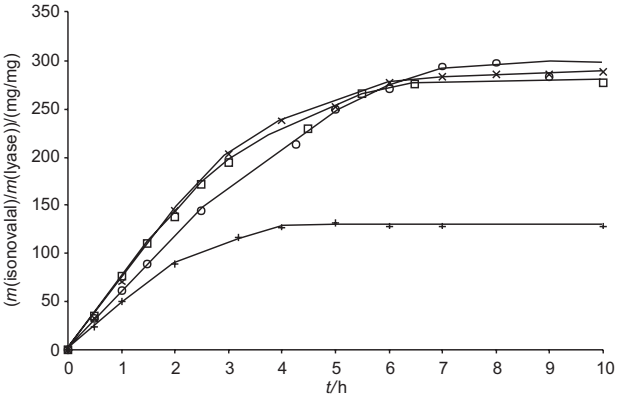

Fig. 3. Evolution with time of isonovalal production performed with purified enzyme (+), biomass supernatant (o), permeabilized biomass (×) and crude extract (□). Bioconversions were carried out with an estimated quantity of 0.15 mg of α-pinene oxide lyase in each fraction. Aqueous phase volume was 2 mL, hexadecane 5 mL. Initial concentration of α-pinene oxide 20 g/L, temperature 30 °C, stirring rate 200 rpm

Thanks to this estimation, it has been possible to compare purified enzyme to other fractions as a biocatalyst for isonovalal production. Results revealed that the purified enzyme appeared to be less efficient for α-pinene oxide bioconversion (Fig. 3). In all cases, a progressive inactivation took place, leading to a reaction stopping at about 6 h. Furthermore, for the same estimated quantity of α-pinene oxide lyase, the reaction rate and the maximal isonovalal concentration obtained were lower with pure enzyme than with other fractions. This result led to consider that a new, specific inactivation phenomenon took place with the purified enzyme.

Interfacial inactivation of α-pinene oxide lyase

A soluble protein assay carried out during bioconversion with purified enzyme indicated a gradual decrease in soluble (active) lyase concentration (Fig. 4). This phenomenon was accompanied by the accumulation of a white precipitate at the water/organic interface. This material was shown to become blue in the presence of Bradford reagent, indicating that it could be considered as denaturated protein.

The enzyme behaviour in the presence or not of an organic layer during 5 h at 30 °C and 200 rpm without substrate was examined. The soluble enzyme concentration was almost constant in a homogenous aqueous system, whereas a rapid decrease in this concentration, comparable to that observed during the course of a bioconversion, was evidenced in the presence of the organic phase (Fig. 4).

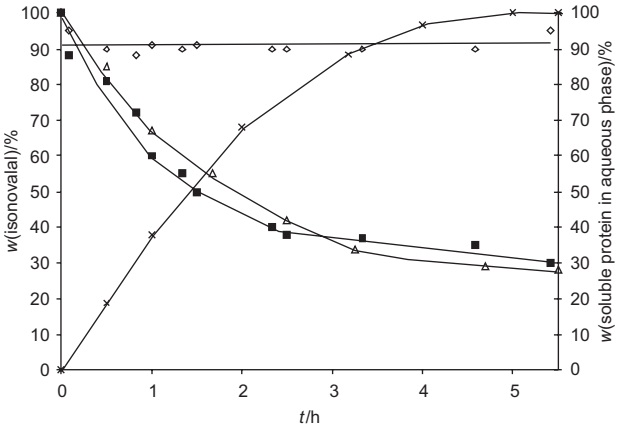

Fig. 4. Evolution of soluble protein concentration during incubation of purified α-pinene oxide lyase solution without precursor and with (■) or without (◊) hexadecane; typical concentrations of α-pinene oxide lyase (△) and of isonovalal (×) during the course of bioconversion carried out with the purified enzyme. Experimental conditions as in the legend of Fig. 3

This decrease in soluble protein content could be related to the loss of enzymatic activity (Table 2). Data in this table also demonstrated that the enzyme was stable in an aqueous medium and that it was protected against hexadecane in the presence of the other bacterial constituents. These results clearly indicate that inactivation of purified enzyme was mainly due to its direct contact with the hexadecane/water interface leading to precipitation. Baldascini and Janssen (*19*) have recently shown a similar inactivation of an epoxide hydrolase at the octane/water interface. Interfacial inactivation can occur by enzyme adsorption to the interface followed by enzyme structural rearrangement (*20,21*). Fig. 5 illustrates a mechanism of enzyme inactivation at the aqueous-organic interface which takes place by unfolding of enzyme molecules adsorbed at the interface, followed by enzyme aggregation and finally precipitation.

Table 2. Behaviour of various enzymatic systems after incubation without a precursor in the presence or not of an organic phase

	Activity/%		
	No incubation time*	5-hour incubation with hexadecane	5-hour incubation without hexadecane
Pure enzyme	100	**0**	100
Permeabilized biomass	100	**93**	88
Biomass supernatant	100	**94**	100

*Reference conditions of bioconversion: the organic layer composed of α-pinene oxide in hexadecane is directly added to the aqueous phase

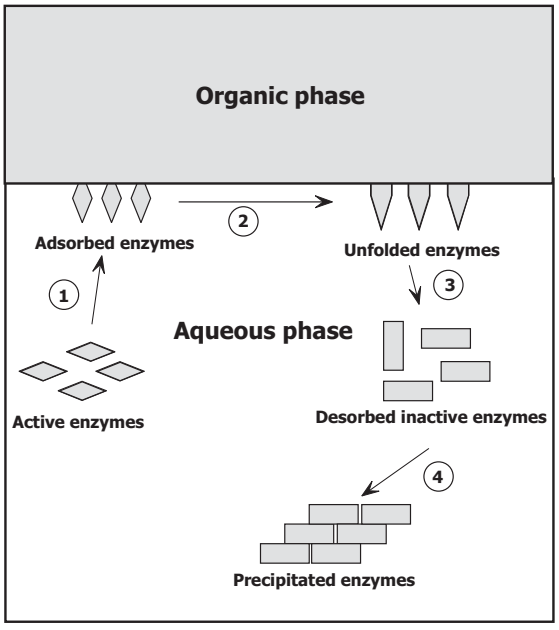

Fig. 5. Schematic representation of enzyme inactivation mechanism at an aqueous/organic interface, adapted from Baldascini and Janssen (*19*). Step 1: reversible enzyme adsorption to the interface and concomitant enzyme structural rearrangement at the interface; step 2: unfolding of enzyme molecule at the interface; step 3: rate-limiting desorption of inactivated/unfolded enzyme molecules from the interface; step 4: irreversible aggregation and precipitation of inactivated enzyme

Protective effect of biomass components

When both permeabilized biomass and biomass supernatant were in contact with hexadecane for 24 hours, no reduction of soluble protein concentration was measured. In all cases, no effect of contact with an organic layer was observed on bioconversion efficiency (data not shown). It could thus be considered that biomass and biomass supernatant contained soluble compound(s) which prevented α-pinene oxide lyase from the water/organic interface denaturation. Among these compounds, membrane components could be eliminated since they were not present in the fraction of biomass supernatant (see Materials and Methods). Thus, this 'protective effect' could be mainly attributed to saturation agents like surfactants or surface proteins which are able to enter the competition for enzyme adsorption at the liquid-liquid interface.

Addition of surfactants like Triton X-100 or Tween 80 at different concentrations to purified enzyme in bioconversion medium had no effect on α-pinene oxide lyase stability (data not shown). More experiments carried out with the addition of bovine serum albumin (BSA/lyase in the ratio of 40:1) allowed reaching the same final concentration of isonovalal as with other fractions (Fig. 6). This protective effect could thus be related to surface proteins, acting as 'sacrificial proteins', which could prevent adsorption of the catalytic enzyme at the interface by a competitive phenomenon (*19*).

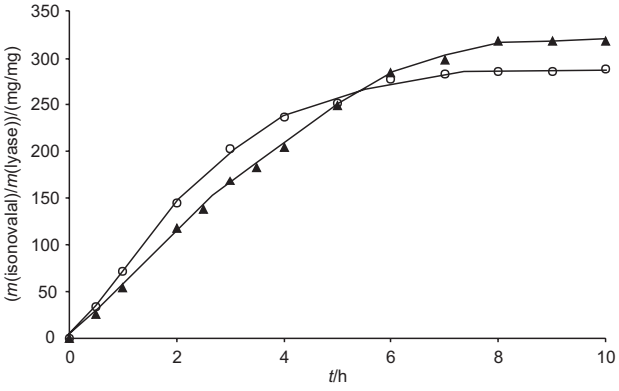

Fig. 6. Time course of isonovalal production with 0.15 mg of purified α-pinene oxide lyase and BSA (3 g/L) (▲), and 0.15 mg of α-pinene oxide lyase estimated in the biomass supernatant (o). Experimental conditions as in Fig. 3

Conclusion

Organic/aqueous biphasic mixtures are generally used to increase productivity of biocatalytic reactions when substrates and/or products are poorly soluble in water. Large interfacial contact areas allow rapid mass transfer between phases. Unfortunately, biphasic systems can also lead to interfacial inactivation of enzymes. This phenomenon has actually been observed in this study with purified α-pinene oxide lyase at the hexadecane/water interface. We suggest that inactivation takes place by unfolding the enzyme molecules adsorbed at the interface, followed by enzyme aggregation and finally precipitation. Purification of α-pinene oxide lyase is then interesting for a better characterisation of the reaction but attracts a limited interest for optimizing isonovalal production. A protective effect of surface proteins produced by the biomass confirms that purification is not interesting for this process. However, a progressive inactivation always takes place, stopping the biocatalytic process. Results obtained here clearly show that inactivation of cellular extracts is not interfacial but can probably be an enzyme turnover due to the catalytic process itself. This point needs further investigations.

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