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ENZYMIC OXIDATION OF ALDEHYDES IN THE ABSENCE OF ADDED ENZYME COFACTORS

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This application is a continuation-in-part of application Ser. No. 329,970 filed Dec. 12, 1963.

This invention relates to enzymic oxidation, and involves the preparation of enzymes from microorganisms and, in particular, the use of the same to oxidize various compounds.

In said application Ser. No. 329,970 there is disclosed and claimed a method for enzymically oxidizing a hydrocarbon in the presence of a sulfhydryl-containing compound as an enzyme activity-enhancing agent, sometimes designated an enzyme cofactor. It has now been found, and it is one of the advantages of the present invention, that enzymic oxidation reactions can be carried out without necessity for the presence of such cofactors.

Another advantage of the invention resides in providing a simplified method of microbially oxidizing various aldehydes to produce carboxylic acids in good yields. Another advantage comprises carrying out such oxidations at good rates. The invention further provides a method of determining and evaluating enzyme activity without resort to conventional gas measurements and analyses. Other advantages will be apparent from the ensuing description.

In brief, the invention comprises mixing an enzyme of a microorganism that is a hydrocarbon oxidizer, as described hereinafter, with the compound to be oxidized, and then incubating the mixture. The oxidized product is suitably recovered from the mixture, or, if the enzyme activity is under study, an examination of the mixture may be made, as described herein, to evaluate such activity. Even though enzyme activity is being studied, oxidized products may be recovered as the amount of the mixture required for the study is negligible.

Considering the invention in detail, the microorganisms with which it is useful are preferably hydrocarbon oxidizers from the genera consisting of Achromobacter, Pseudomonas, Nocardia, Bacillus, and Mycobacterium, including such species as *A. xerosis, A. gutatus, A. superficialis, A. Parvulus, A. cycloclastes, Ps. aeruginosa, Ps. oleovorans, Ps. putida, Ps. fluorescens, Ps. boreopolis, Ps. methanica, N. corallinus, N. opacus, N. paraffinae, N. salmonicolor, B. hexacarbovorum, B. mesentericus, B. toluolicum, M. phlei, M. rubrum, M. luteum, M. lacticola, M. album, M. byalinicum,* and *M. laprae.* A preferred class comprises the Achromobacter.

A wide variety of aldehydes may be enzymically oxidized according to the invention. These comprise aliphatic, aromatic, alicyclic and heterocyclic compounds. The aliphatic aldehydes may be saturated or unsaturated and have straight or branched chains; suitably they have up to 20 or 30 carbon atoms per molecule. The cyclic compounds may be mono or polynuclear compounds; may be substituted, as by alkyl groups, or may not; and may have the aldehyde group in the side chain or the ring. In all of the compounds, more than one aldehyde group may be present. Preferred compounds are alkanals and alkenals having 1 to 20 carbons and with a terminal aldehyde group, such as propanal, acrolein, malonaldehyde, butanal, crotonaldehyde, pentanal, glutaconaldehyde, hexanal, heptanal, octanal, nonanal, decanal, dodecanal, octadecanal, propenal, butanedial, butenedial, hydroxybutanal, etc. Other useful aldehydes include aromatic aldehydes like benzaldehyde, tolualdehyde, cuminal, phthalaldehyde, salicylaldehyde, anisaldehyde, phenylacetaldehyde, cinnamaldehyde, 1-naphthaldehyde, 2-anthraldehyde, etc. Suitable alicyclic compounds include tetrahydrobenzaldehyde and similar 6-ring and 5-ring compounds. Heterocyclic aldehydes include furfural, cinchoninaldehyde, nicotinaldehyde, etc.

Cells of the microorganism from which the enzyme is derived may be grown in an agitated system using a hydrocarbon as the sole source of carbon. In addition, the system or culture also contains a medium comprising conventional mineral salts, including a source of nitrogen such as sodium nitrate or ammonium sulfate. Preferred pH of the system is 5.5 to 8.5.

The hydrocarbon which forms the sole source of carbon for the cells may be aliphatic or cyclic, the latter including aromatic and alicyclic compounds. The aliphatic hydrocarbon is saturated or unsaturated, straight or branched chain, and has up to 20 to 30 or more carbon atoms. Saturated straight chain alkanes having up to 20 carbons are preferred, particularly those which are liquid at the temperatures employed.

The cyclic hydrocarbon may be an aromatic, or a saturated or unsaturated 5- or 6-membered cycloparaffin; it may have one or two or more rings; and is preferably an alkyl-substituted cyclic hydrocarbon having one, two, or more alkyl substituents, each of any suitable length, and comprising straight or branched chain radicals.

Alkyl-substituted aromatic hydrocarbons are preferred, and include toluene, the various xylenes, mesitylene, ethylbenzene, p-cymene, the diethylbenzenes, and the isomeric propylbenzenes, butylbenzenes, amylbenzenes, hexylbenzenes, heptylbenzenes, and octylbenzenes. Though unsubstituted, benzene is suitable.

Included among the alkyl-substituted cycloparaffins are methylcyclopentane, the di- and trimethylcyclopentanes, the ethyl- and diethylcyclopentanes, the various propyl-, butyl-, amyl-, hexyl-, heptyl-, and octylcyclopentanes. Also included are alkylcyclohexanes which are substitued in a manner corresponding to the foregoing alkylcyclopentanes, and further including such compounds as the various tetramethyl-, methylethyl-, and methylpropylcyclohexanes, and the like. Unsubstituted cyclopentane and cyclohexane are useful.

The cells are harvested while they are young and rapidly dividing, i.e., while they are in the phase of exponential growth. This phase, it will be recalled, follows the lag phase and precedes the maximum stationary phase; in the former, a constant growth rate is not yet established, while in the latter, growth ceases. But in the exponential or logarithmic phase, cell growth proceeds at a constant rate. In ordinary liquid cultures, this latter phase lasts only for a relatively short period of time, and it is therefore important to follow or control the system or culture so as to be able to harvest the cells at the proper time. A suitable procedure for determining the desired harvesting time is to limit the amount of one or more of the nutrients in the system, such as the nitrogen or carbon, preferably the source of carbon, so that when this component is exhausted, growth of the cells will cease while they are still in the exponential phase, i.e., while they are still young, after which they are quickly harvested. Suitable methods are available for determining the presence of the carbon source compound in the system, including the physical separation of such compound from an aliquot sample of the aqueous culture; or a conventional chemical test to determine its presence; or, the running of trial or calibration incubations to obtain data for constructing a concentration versus time plot by means of which, in subsequent incubations, the concentration of carbon compound may be determined simply by a knowledge of the time or duration of the incubation.

A preferred method for determining the presence of the carbon source compound comprises employing a dye such as a tetrazolium salt. This material is an electron acceptor, and in its oxidized state it is colorless and water soluble, but as it is reduced it becomes colored and insoluble in water. Assuming that the sole source of carbon in the system is decane, the procedure comprises drawing two aliquot portions or samples from the fermenter or culture, adding the dye to the first aliquot, and adding the dye plus a small amount of decane to the second. The optical density of the two aliquots is then measured. The optical density of the second aliquot will be appreciable because decane is present to be oxidized by the cells, and as it is oxidized, the dye is reduced, becoming colored and insoluble, such that in the optical density test it will adsorb the radiation employed and give a substantial adsorbancy value. In the first aliquot, if the decane has been all or substantially all used up, there will be none present to be oxidized by the cells, and therefore no dye will be correspondingly reduced to the colored form, and in turn the optical density value will be low by comparison with that of the second aliquot, which serves as a control. Thus, according to this method the desired cell harvesting time is determined by an easily run optical density test.

The tetrazolium salt is a stable, water-soluble, organic salt, functioning as a cationic indicator which undergoes reduction as the carbon source compound is oxidized and which does not readily reoxidize in the presence of oxygen. More particularly, these salts function as electron acceptors; during oxidation of the carbon source compound, electrons are released and the electron acceptor, as the name implies, takes the released electrons. Suitable tetrazolium salts include 2,3,5-triphenyltetrazolium chloride, neotetrazolium, blue tetrazolium, tetrazolium violet, iodonitrotetrazolium chloride, and others.

The optical density test referred to may be a spectrophotometric test such as one involving the selective adsorption of infrared rays, or one involving the adsorption of visible light rays of a suitable wave length. As indicated, as the tetrazolium salt is reduced, it becomes insoluble in water and in addition acquires a distinctive color, and in this form it will adsorb radiation, the amount of which is measured.

It is desirable, before harvesting, that the cells in the phase of exponential growth exhibit an optical density value in the range of 0.2 to 16, preferably 0.4 to 6, optical density units. In terms of the dry weight of the cells, the broader of these ranges corresponds to 50 to 4000 mg. of cells per liter of aqueous culture, while the narrower range corresponds to 100 to 1500 mg. of cells, per liter. As is apparent, one optical density unit is equivalent to 250 mg. of cells per liter.

Following harvesting, the cells are washed, and then suspended in an aqueous medium and ruptured to free or make available intracellular enzyme components thereof. Cell rupture may be done by exposing the cells to high frequency oscillations or by other conventional methods including grinding in the presence of abrasives, shaking with abrasives, exposure to lysozyme, compressing and release of pressure as in a French pressure cell, or other physical-chemical treatments designed to rupture the cell and release soluble components therefrom.

The procedure for enzymically oxidizing an aldehyde is illustrated in the examples below but may be described briefly as follows. The selected microorganism is grown in a culture medium comprising the mineral salts, nitrogen source, and a hydrocarbon as the sole source of carbon. The cells are harvested in the exponential growth phase, the soluble enzyme extract is removed from them as described, this extract is then mixed with the aldehyde to be oxidized, and the mixture is incubated. Incubation may be carried out at about 20 to 55° C., preferably around 30° C., and for times generally extending up to several hours. During incubation, the mixture is preferably stirred and

is maintained accessible to air. Its pH is preferably in the range of 6 to 9.

At the conclusion of the oxidation, a small amount of strong mineral acid may be added to stop the reaction. The oxidized products are recovered, suitably by extraction of the incubation mixture with a conventional solvent such as ether, and after evaporation of the ether from the extract, the products may be further separated and purified by conventional procedures such as distillation, fractional crystallization, and the like.

It may be noted that the hydrocarbon upon which the cells are initially grown may be different from the aldehyde which is incubated with the bacterial extract, although preferably it is similar, i.e., has a similar carbon configuration. In other words, aliphatic hydrocarbons are preferred where the aldehyde is aliphatic, aromatic hydrocarbons where the aldehyde is aromatic, etc. More preferably, the hydrocarbon has substantially the same carbon configuration as the aldehyde, and/or substantially the same number of carbon atoms. But while similarity is preferred, it is, as indicated and as will be understood, not essential, as illustrated by the fact that cells grown on a hydrocarbon like p-cymene will yield an enzyme extract that can enzymically oxidize an aldehyde like decanal.

The oxidized product is a carboxylic acid having the same number of carbons and the same carbon skeleton as the aldehyde. Thus, n-decanal yields decanoic acid, p-isoproply benzaldehyde yields p-isoproply benzoic acid, and so forth. Good enzymic oxidation rates for producing the product are obtainable by virtue of the fact that the enzyme extract is derived from growing cells in the exponential growth phase. Furthermore, the addition of an enzyme cofactor, such as a sulfhydryl-containing compound, to the bacterial enzyme mixture undergoing incubation is not found to be necessary.

To study the activity of an enzyme extracted from the microorganisms, the selected cells are grown, harvested while in the exponential phase, then ruptured to free the soluble enzyme material, and the latter is mixed with the aldehyde and also with a suitable indicator for following or assaying the ensuing oxidation reaction, such as a tetrazolium salt. The resulting mixture of enzyme extract, aldehyde, and indicator is then incubated. However, instead of, or in addition to, recovering the oxidation products, a small amount of the mixture is subjected to an optical density test of the kind described to quickly determine the extent of oxidation and thus to provide information on the enzyme activity. By comparing the adsorbency of the mixture with that of a control, the extent of reduction of the tetrazolium salt may be obtained, which in turn corresponds to the extent of oxidation of the aldehyde, and in turn to the enzyme activity.

For the enzymic oxidation of an aldehyde, and on the basis of 1 ml. of the mixture which is incubated, the amount of bacterial extract is 0.1 to 60, preferably 3 to 10, mg. of protein; and the amount of aldehyde is 0.1 to 30, preferably 10 to 30, micromoles. When a tetrazolium salt is used for determination of enzyme activity, the amount is 0.01 to 10, preferably 0.1 to 0.5, micromoles. As is apparent, the amount of the bacterial extract is given in terms of its protein content. It will be understood that in each ml. of the foregoing mixture, water is also present. A suitable method of bringing together the bacterial extract and the aldehyde is to first dissolve or disperse each in water and then to combine these aqueous portions. In the case of water-insoluble aldehydes, a dispersion or emulsion of the same may be prepared and then added to the bacterial extract, or a common solvent is used, such as a low molecular weight alkanol.

The invention may be illustrated by the following examples.

EXAMPLE 1

An organism identified as a member of the genus Achromobacter was grown in a 5-liter fermenter on a con-

ventional mineral salts medium using ammonium sulfate and urea as the source of nitrogen. The medium comprised the following, per liter of solution:

	G.
Ammonium sulfate	1.0
Potassium dihydrogen phosphate	2.0
Sodium monohydrogen phosphate	3.0
Magnesium sulfate	0.2
Calcium chloride	0.01
Ferrous sulfate	0.005
Manganese sulfate	0.002
Sodium carbonate	0.1
Urea	1.5

A total of 3 liters of culture was present in the fermenter. As the sole source of carbon, an amount of n-decane was added which would limit the growth of the organism by becoming exhausted when the cells were in the exponential growth phase. The amount chosen was 5 ml. As soon as the cells had substantially exhausted the decane present, one liter of the aqueous culture was removed for cell harvesting and an extract of the soluble enzymes was prepared. To the cells remaining in the fermenter an excess of n-decane was added, about 10 ml., and they were allowed to grow to a maximal density, i.e., to the maximum stationary phase, after which they were harvested and an extract prepared.

The procedure for preparing each extract comprised harvesting the cells by centrifugation, washing them with distilled water, and then suspending them in distilled water to provide a concentration of about 1 g. of cells per 4 ml. of suspension. This suspension was then exposed to the high frequency oscillations of an ultrasonic device identified as a Branson Sonofier to rupture the cells and to free soluble cell components, including enzymes, which then dissolved in the distilled water. Cell debris was removed from the solution by centrifugation as 34,000 times gravity.

The two extracts, comprising that from the young growing cells and that from the old cells in their maximum stationary phase, were compared for their ability to oxidize decanal. For comparative purposes, the effect of the presence of a cofactor, or enzyme activity-enhancing agent, was also studied, the cofactor being cysteine. A tetrazolium salt (2,3,5-triphenyltetrazolium chloride) was added to each test mixture to follow the reaction. Four test mixures were prepared: No. 1 comprising the extract solution from the young cells, decanal, and the tetrazolium salt; No. 2 being the same as No. 1 except that it contained cysteine; No. 3 comprising the extract solution from the old cells, decanal, and the tetrazolium salt; and No. 4 being the same as No. 3 but also containing cysteine. Into each mixture nitrogen was run for about 15 seconds, after which the mixture was stoppered and incubated for 15 minutes at 25° C., with shaking. In each case air was also present. The oxidation of the decanal was stopped by the addition of 0.1 ml. of 3 N sulfuric acid and 3 ml. of ethanol. Each mixture, after centrifuging to remove any solids, was then tested for adsorption of visible light rays of a wave length of 500 millimicrons (0.5 micron) in a Bausch and Lomb colorimeter, and from the resulting values the amounts of decanal oxidized were calculated. The data follows:

Extract from—	Decanal Oxidation, micromoles oxidized	
	Without Cysteine	With Cysteine
Old cells	0.053	0.17
Young cells	.333	.24

All values were corrected by subtracting the level of activity in the absence of the decanal. It will be seen that the enzyme extract from the young cells produced the greater amounts of oxidized decanal, and that the young cells had better activity without cysteine.

EXAMPLE 2

An enzyme extract was prepared from young cells in the exponential growth phase, as described in Example 1. It comprised 1 ml. of an aqueous solution, pH about 8, protein content about 3 to 5 mg. To this small scale system there was added 100 micromoles of decanal and the mixture was incubated for three hours with the system having access to air. The mixture was then extracted with ether and the ether extract separated. After evaporation of the ether, a solid product was recovered and this was identified as decanoic acid by vapor phase chromatography of the methyl ester. The methyl ester of a known sample of decanoic acid was used as a control in the latter step.

It will be understood that the invention is capable of obvious variations without departing from its scope.

In the light of the foregoing description, the following is claimed.

1. Method for microbiologically oxidizing an aldehyde which comprises growing cells of a microorganism in a fermentation mixture comprising a hydrocarbon as the sole source of carbon and a medium capable of supporting growth of said microorganism, said microorganism being a hydrocarbon oxidizer and being selected from the class consisting of hydrocarbon-oxidizing species of Achromobacter, Pseudomonas, Nocardia, Bacillus, and Mycobacterium, harvesting the cells of the microorganism while in the phase of exponential growth and washing the same, suspending the cells in an aqueous medium and rupturing the same, thereby dissolving soluble enzymes thereof in said aqueous medium, then adding to the aqueous medium said aldehyde to be oxidized, and incubating the resulting mixture to oxidize the aldehyde to its corresponding carboxylic acid.

2. Method of claim 1 in which said microorganism is selected from hydrocarbon-oxidizing species of Achromobacter.

3. Method of claim 1 in which said microorganism is selected from hydrocarbon-oxidizing species of Pseudomonas.

4. Method of claim 1 in which said microorganism is selected from hydrocarbon-oxidizing species of Nocardia.

5. Method of claim 1 in which said microorganism is selected from hydrocarbon-oxidizing species of Bacillus.

6. Method of claim 1 in which said microorganism is selected from hydrocarbon-oxidizing species of Mycobacterium.

7. Method of claim 1 wherein said hydrocarbon has substantially the same carbon configuration as said aldehyde.

8. Method of claim 1 wherein the amount of a nutrient for the cells is restricted so that the cells exhaust said amount of nutrient while in the phase of exponential growth, thereby insuring that the cells for the harvesting step are young growing cells.

9. Method for microbiologically oxidizing an aldehyde which comprises growing cells of a microorganism in a fermentation mixture comprising a hydrocarbon as the sole source of carbon and a medium capable of supporting growth of said microorganism, said microorganism being a hydrocarbon oxidizer and being selected from the class consisting of hydrocarbon-oxidizing species of Achromobacter, Pseudomonas, Nocardia, Bacillus, and Mycobacterium, restricting the amount of a nutrient for the cells so that the cells exhaust said amount of nutrient

while in the phase of exponential growth, harvesting the cells of the microorganism while in said phase of exponential growth, suspending the cells in an aqueous medium and rupturing the same, thereby dissolving soluble enzymes thereof in said aqueous medium, then adding to the aqueous medium said aldehyde to be oxidized, incubating the resulting mixture in the absence of added enzyme cofactors, and forming therein a carboxylic acid as the oxidation product of said aldehyde.

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