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Enzymic routes to dihydroxyacetone phosphate or immediate precursors

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Abstract

Dihydroxyacetone phosphate (DHAP) is an important substrate for enzymic syntheses of saccharides by means of dihydroxyacetone phosphate aldolases. A convenient precursor of DHAP is L-glycerol-3-phosphate (L-G3P), the enzymic synthesis of which may be achieved by phosphate transfer owing to an ATP-regenerating system. The phosphorylation of both glycerol and dihydroxyacetone by means of an ATP-regenerating system based on phosphoenolpyruvate, pyruvate kinase and glycerol kinase is examined in order to optimize the reaction conditions. The composition of the reaction mixture should not exceed concentrations of 300 mM of each phosphoenolpyruvate and glycerol. Otherwise, the productivity would be affected by substrate inhibition. An operating temperature of 50 °C may be applied, when the enzymes should be used only once. At a temperature of 35 °C, a high half-life of both enzymes glycerol kinase and pyruvate kinase is achieved. Various concentrations of the enzymes are tested to optimize the use of the enzymatic activity. A ratio of 2:3 of pyruvate kinase and glycerol kinase activity seems to be adequate. Further studies show that 10 mM ATP is enough to maintain a permanent supply of the cofactor. It is shown, how DHAP may be obtained from L-G3P by means of an L-G3P oxidase and catalase, the latter being used not only to decompose the hydrogen peroxide formed during the course of reaction, but also to supply the reaction system with oxygen. This is achieved by feeding a H_2O_2 solution in a way to keep the oxygen level constant in the reaction mixture.

Keywords: Dihydroxyacetone phosphate; L-Glycerol-3-phosphate; Phosphoenolpyruvate; Glycerol kinase; Pyruvate kinase

1. Introduction

Dihydroxyacetone phosphate-dependent aldolases catalyze the condensation of quite a number of different aldehydes with dihydroxyacetone phosphate (DHAP) to yield mostly unusual carbohydrates. A complete set of four known DHAP aldolases of complementary specificity with respect to the stereochemistry of the two C-atoms connected by the newly formed C–C bond are known [1–4]. DHAP is mandatory for these reactions, and the need for DHAP in large quantities requires efficient syntheses to be worked out.

There are numerous chemical as well as biocatalytic routes for the production of DHAP [5]. However, most of the methods yield complex substrate mixtures containing phosphate ions as well as phosphorylated substances other than DHAP. These by-products are hard to be separated from DHAP and often have a strongly inhibitory affect on aldolases [6]. Therefore, a well known purely enzymatic route [7] with the stable intermediate L-glycerol-3-phosphate (L-G3P) seems to be optimal in order to obtain DHAP in high quality.

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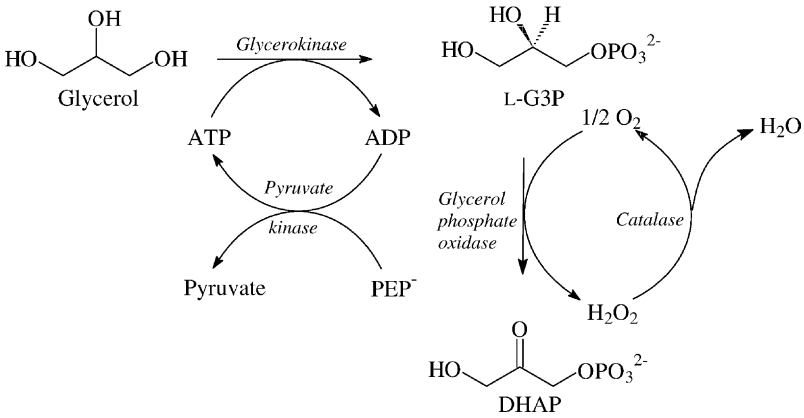

Fig. 1. Synthesis of dihydroxyacetone phosphate (DHAP) from glycerol with glycerokinase and glycerol-3-phosphate oxidase (GPO).

This route for gaining DHAP is summarized in Fig. 1 and includes the transformation of L-G3P into DHAP by means of a G3P oxidase [8].

The optimization of the different operating variables responsible for the synthesis was studied and is treated in this article. Another important aspect is the instability of DHAP with respect to temperature and pH. The decay follows the reaction scheme published by Richard [9].

2. Experimental

Chemicals were reagent grade and used without further purification. ATP (art. no. 1068), NADH (1051) and NAD^+ (1013) were purchased from Gerbu (D). Glycerol (4094) and magnesium chloride (5833) were from Merck (D). *o*-Dianisidine (33435) was from Fluka (D, CH) and dihydroxyacetone (DHAT-EIS-001) from Daicel (J). Phosphoenolpyruvate was prepared according to the method of Hirschbein et al. [10].

Peroxidase type I from horseradish (P-8375) was obtained from Sigma. All other enzymes were kindly supplied by Boehringer Mannheim (D), now Roche (D,CH): catalase from bovine liver (106836); glycerol-3-phosphate dehydrogenase from rabbit muscle (84012221); glycerokinase from *Bacillus stearothermophilus* (83439629); L-glycerol-3-phosphate oxidase from microorganisms (775797); L-glycerol-3-phosphate oxidase from microorganisms, stabilized, lyophilized, 7.64 U/mg; lactate dehydrogenase from bovine heart (14199723); pyruvate kinase from rabbit muscle (83486721).

Spectrophotometric measurements were performed at 25 °C using a Pharmacia Biochrom Model 4060 spectrophotometer equipped with a constant temperature cell compartment.

2.1. Inorganic phosphate

Phosphate concentrations were measured using a Sigma test kit (360-3) for determining inorganic phosphate.

2.2. Dihydroxyacetone phosphate

DHAP concentrations were determined enzymatically. The conversion of DHAP to L-G3P catalyzed by glycerol-3-phosphate dehydrogenase was coupled with the consumption of NADH, which was followed spectrophotometrically by recording the absorption at a wavelength of 360 nm [6].

2.3. L-Glycerol-3-phosphate

L-G3P concentrations were measured on the basis of the oxidation of L-G3P to DHAP catalyzed with L-glycerol-3-phosphate oxidase (GPO). Thus H_2O_2 was generated, which led to oxidize *o*-dianisidine in the presence of a peroxidase (POD). This gave a colored complex, the absorption of which was monitored spectrophotometrically at a wavelength of 460 nm [6].

2.4. Determination of enzyme activities

The activity of pyruvate kinase was determined by coupling the main reaction with the transformation of pyruvate to lactate catalyzed by lactate dehydrogenase. The disappearance of NADH was followed spectrophotometrically at a wavelength of 340 nm at a pH of 7.1 and 25 °C [6].

The activity of glycerokinase was determined by coupling the main reaction with the transformation of L-G3P to DHAP catalyzed by glycerol-3-phosphate dehydrogenase. The appearance of NADH was followed spectrophotometrically at a wavelength of 340 nm at a pH of 9.8 and 25 °C [6].

The activity of glycerol-3-phosphate oxidase was determined by coupling the main reaction with the peroxidase catalyzed oxidation of *o*-dianisidine the product of which was followed spectrophotometrically at a wavelength of 430 nm at a pH of 7.5 and 25 °C [8].

2.5. Biotransformation experiments

The phosphorylation of glycerol was performed in jacketed glass vessels with a working volume of 50 ml (titration vessels 6.1415.22X of Metrohm, CH), the substrate solution in which was agitated by a magnetic stirrer (Metrohm 649) and kept at the desired temperature by means of a thermostat (Julabo FC HC). The reaction was started by the final addition of glycerokinase into the equilibrated substrate solution.

Batch-reactor experiments with recovery of the enzymes glycerokinase and pyruvate kinase by means of ultrafiltration were carried out in a thermostatted ultrafiltration device (XF UF-07601, Millipore, USA), a magnetically stirred cell equipped with a flat membrane of regenerated cellulose with a nominal cut-off of 10,000 Da (PLG 07610, Millipore, USA). The reaction was carried out as usual prior to closing the stirred cell and changing to ultrafiltration mode of operation by providing nitrogen gas under a differential pressure of 1 bar to the head space of the ultrafiltration device. Ultrafiltration was performed until a small residual volume of the reaction medium was achieved, which was immediately filled up with fresh substrate solution starting a new cycle of batch reaction.

The oxidation of L-G3P was performed in a jacketed glass vessel with a working volume of 350 ml equipped with magnetic stirring, an oxygen electrode (transmitter 301 and microsensor of UMS, D), and a supply of a 2 M oxygen peroxide solution by means of an automatic burette of 1 or 5 ml (Dosimat 665, Metrohm, CH). A feedback control for keeping the oxygen concentration in the reaction solution constant by feeding the hydrogen peroxide solution was achieved by a personal computer equipped with data acquisition hardware operating under Labtech Control (Labtech, USA).

3. Results and discussion

A major problem with dihydroxyacetone phosphate is its instability in aqueous environment. This may limit the operating variables for the production of DHAP considerably. Thus, Figs. 2 and 3 give an impression upon the decomposition of DHAP in aqueous solution as a function of temperature and pH, respectively.

Low pH and/or temperatures lower than 35 °C are absolutely required, if long operating times have to be accepted due to economic reasons with respect to enzyme costs. This limits the choice of operating conditions significantly. In addition, most of the common enzymes of interest for the production of DHAP have their pH-optimum in the neutral or the alkaline pH range. This is a particular restriction for optimizing the reaction. It should be mentioned that a novel synthetic procedure has been published, in which only racemic glycerol-3-phosphate can be produced at pH 4 by means of a phytase in the presence of pyrophosphate. The conversion to DHAP has to be achieved by means of the L-glycerol-3-phosphate oxidase as described here [11,12].

4. Synthesis of L-glycerol-3-phosphate

The phosphorylation of glycerol by glycerokinase is a very efficient route for the production of L-G3P—especially, when phosphoenolpyruvate (PEP) and pyruvate kinase (PK) are applied as the ATP-regenerating system. PEP is readily available by chemical synthesis [10]. The glycerokinase from *Bacillus stearothermophilus* phosphorylates glycerol much more readily than dihydroxyacetone—as shown in Fig. 4. Here, a pH of 6.5 has been applied in order

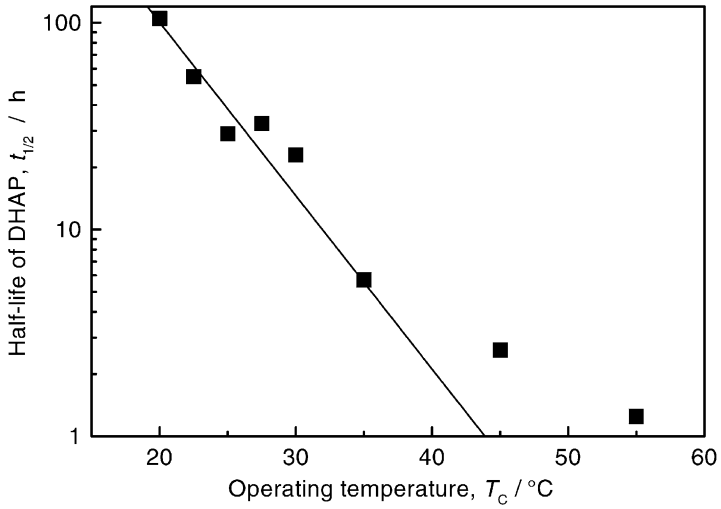

Fig. 2. Temperature dependence of the chemical stability of DHAP in aqueous solution. Initial conditions: $c_{DHAP} = 80$ mM; pH = 6.8.

to be able to operate under conditions under which DHAP is fairly stable.

The glycerokinase exhibits higher activity at pH 9. Since L-G3P is still quite stable under this pH, L-G3P may be obtained under much more favorable conditions than DHAP. Therefore, L-G3P is more readily available by means of this classical route. The temperature dependence of the L-G3P production with the GK/PK system may be derived from Fig. 5 for one particular substrate concentration.

A whole series of similar experiments have been performed at different substrate levels. The results are gathered in Fig. 6. The time course of the reactions—as given in Fig. 5—shows almost a zero-order behavior. Since L-G3P is a rather stable substance, optimum conditions with respect to pH and temperature may be chosen with much less restriction. Thus, an operating pH of 9 and a temperature of 50 °C may be applied, when the enzymes are supposed to be used only once.

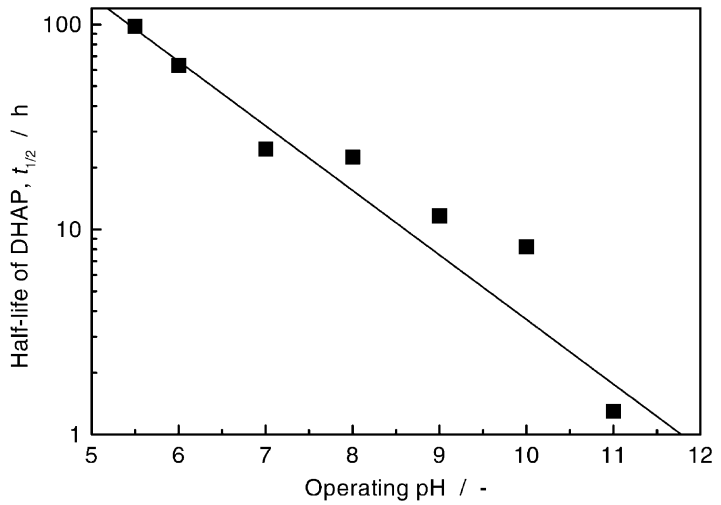

Fig. 3. pH dependence of the chemical stability of DHAP in aqueous solution. Initial conditions: $c_{DHAP} = 80$ mM; $T_C = 25$ °C.

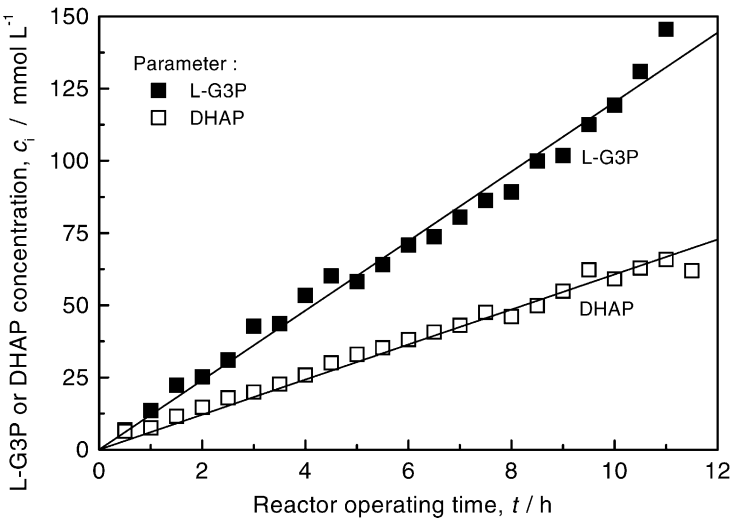

Fig. 4. Syntheses of L-glycerol-3-phosphate (L-G3P) and dihydroxyacetone phosphate (DHAP) by means of the ATP-regenerating system based on glycerokinase (GK) and pyruvate kinase (PK). Initial reaction conditions: $c_{PEP} = 300\,mM$, $c_{ATP} = 2.5\,mM$, $c_{MgCl_2} = 2.5\,mM$, $E_{GK} = 2\,kU\,l^{-1}$, $E_{PK} = 8\,kU\,l^{-1}$; for L-G3P: $c_{Gly} = 300\,mM$; for DHAP: $c_{DHA} = 300\,mM$; pH = 6.5, $T_C = 25\,°C$.

Substrate concentrations higher than 300 mM are not recommended due to substrate inhibition of the system GK/PK (data not shown). A ratio of 3:2 for the activities of PK and GK showed to be sufficient, in order to provide GK with enough ATP, and not to waste precious activity of GK.

The ATP concentration should also be reduced to a minimum in order to minimize the production costs of DHAP. Fig. 7 shows the rate of reaction as a function of the ATP concentration. It shows the characteristics of a Michaelis–Menten kinetics. A concentration of 10 mM ATP seems to be

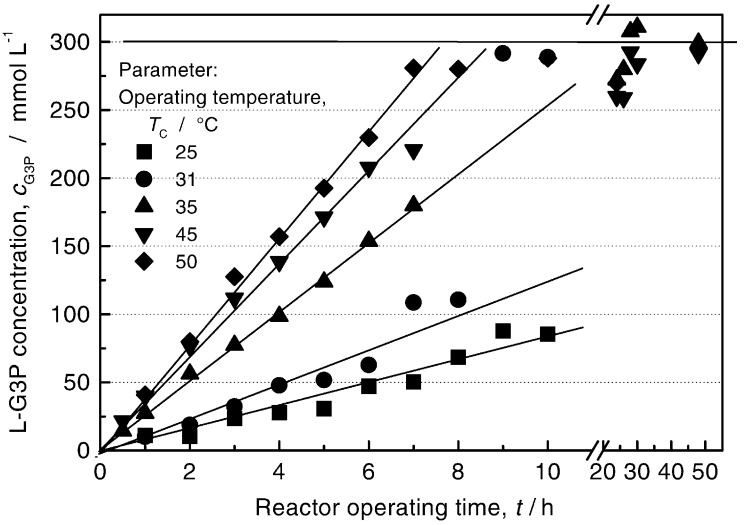

Fig. 5. Syntheses of L-glycerol-3-phosphate (L-G3P) by means of the ATP-regenerating system based on glycerokinase (GK) and pyruvate kinase (PK) at different operating temperatures. Initial reaction conditions: $c_{PEP} = 300\,mM$, $c_{Gly} = 300\,mM$, $c_{ATP} = 10\,mM$, $c_{MgCl_2} = 10\,mM$, $E_{GK} = 4\,kU\,l^{-1}$, $E_{PK} = 4\,kU\,l^{-1}$, pH = 9.

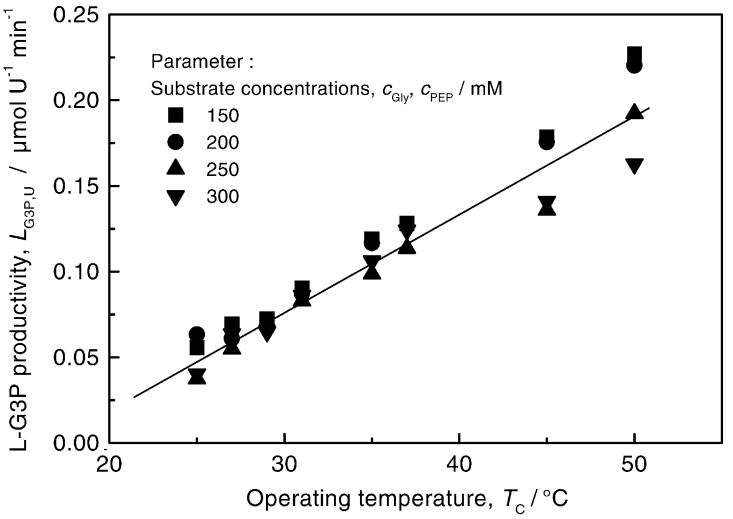

Fig. 6. Temperature profile for the phosphorylation of glycerol. Initial reaction conditions: $c_{ATP} = 10\,mM$, $c_{MgCl_2} = 10\,mM$, $E_{GK} = 4\,kU\,l^{-1}$, $E_{PK} = 4\,kU\,l^{-1}$, pH = 9.

sufficient for supplying the system with enough cofactor.

The choice of the operating conditions would be less flexible, if the enzymes should need to be recovered. The repeated use of the enzymes would require that the activity should remain for some reaction cycles. Thus, some repetitive batch operations have been performed with intermittent recovery of the enzymes by means of ultrafiltration. The reactor was operated at different temperatures. The results are summarized in Table 1. In the case of enzyme recycling, the operating temperature should obviously not exceed 35 °C, if a batch reaction cycle time of 20 h is applied.

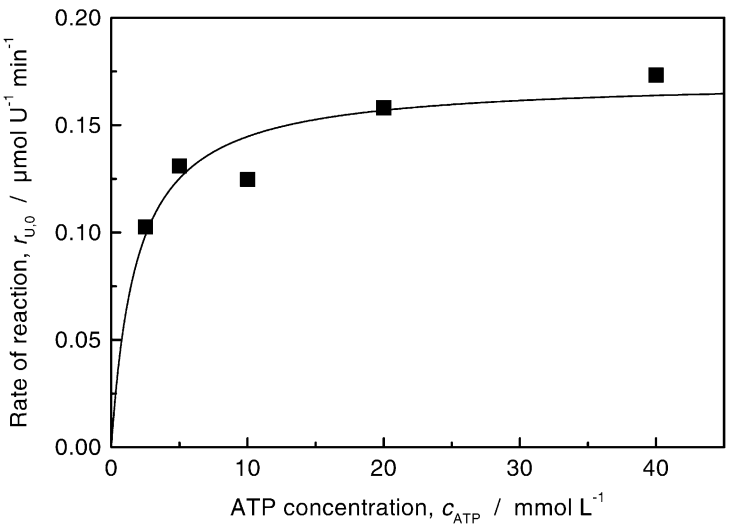

Fig. 7. Kinetics of the production of L-glycerol-3-phosphate (L-G3P) by means of the ATP-regenerating system based on glycerokinase (GK) and pyruvate kinase (PK) at 50 °C. Initial reaction conditions: $c_{PEP} = 300\,mM$, $c_{Gly} = 300\,mM$, $c_{MgCl_2} = 10\,mM$, $E_{GK} = 8\,kU\,l^{-1}$, $E_{PK} = 8\,kU\,l^{-1}$, pH = 9, $T_C = 50\,°C$.

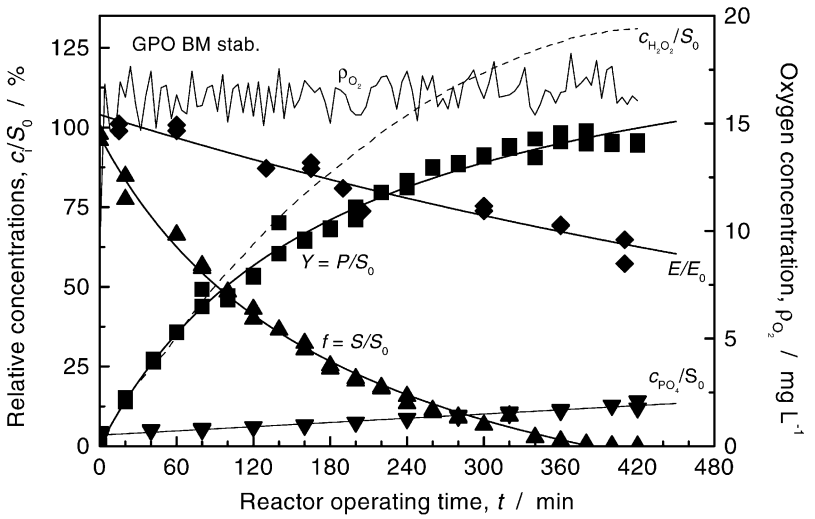

Fig. 8. Production of dihydroxyacetone phosphate (DHAP) from L-G3P by means of a stabilized G3P oxidase by feeding a 2 M hydrogen peroxide solution in response to feedback control of the oxygen concentration in the medium of 15.8 mg l^{-1}. Initial conditions: $c_{G3P} = 100$ mM, pH = 7, $T_C = 27\,^{\circ}$C.

Table 1
Yields of L-glycerol-3-phosphate (L-G3P) in a repeated batch-reactor operation with enzyme recovery by means of ultrafiltration

T_C (°C)	Yield of L-G3P after a reaction time of 20 h (%)				
	1[a]	2[a]	3[a]	4[a]	5[a]
35	91	86	79	75	36
45	81	82	25	28	
50	79	78	38		
55	100	16			

Initial reaction conditions: $c_{PEP} = 300$ mM, $c_{Gly} = 300$ mM, $c_{ATP} = 10$ mM, $c_{MgCl_2} = 10$ mM, $E_{GK} = 4$ kU l^{-1}, $E_{PK} = 4$ kU l^{-1}, pH = 9.

[a] Cycle number.

5. Synthesis of dihydroxyacetone phosphate

The conversion of L-glycerol-3-phosphate to dihydroxyacetone phosphate is conveniently performed by glycerol-3-phosphate oxidase. The oxygen required may be supplied through membrane aeration because of stability problems of GPO in the presence of air bubbles [8]. However, the oxygen supply is achieved preferably in the presence of hydrogen peroxide and catalase. The concentration of hydrogen peroxide is kept very low by feeding a hydrogen peroxide solution in order to keep the oxygen saturation constant in the reaction mixture. An example of such a batch-reactor experiment is given in Fig. 8. The concentrations of the different reactants are given as quantities relative to the initial substrate concentration (S_0). Oxygen is generated at a supersaturation level of 15.8 mg l^{-1} by controlled feeding of a 2 M H_2O_2 solution. Under these conditions, apparently only 25% of the oxygen required is lost. The concentration of phosphate ions generated by the decomposition of DHAP is low, although a long operating time has to be used in order to achieve a high DHAP yield (Y). The residual activity of the enzyme GPO (E/E_0) is about 60% after 7 h. Thus, this technique is quite convenient and easy for scale up.

6. Conclusion

Dihydroxyacetone phosphate may readily be obtained by means of the classical route from glycerol by means of a glycerokinase-pyruvate kinase ATP-regenerating system leading first to L-glycerol-3-phosphate as a stable intermediate, which may be converted to DHAP prior to its use by means of a G3P oxidase. This strategy leads to a better usage of the enzymes involved. Thus, a high operating temperature (50 °C)

and a high pH (9) can be chosen for the production of L-G3P. The use of hydrogen peroxide as oxygen supply for the oxidase reaction is very convenient, since catalase has to be present anyway.

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