INFLUENCE OF TEMPERATURE ON BIOCONVERSION OF D-GLUCOSE INTO 2-KETO-D-GLUCOSE BY PYRANOSE OXIDASE

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The enzymatic reactions of high selectivity and specificity are new bioengineering alternatives for obtaining a wide variety of food and pharmaceutical products. In this context, the regiospecific oxidation at the pyranose C2 position catalyzed by pyranose oxidase (P2Ox) is a very useful reaction for carbohydrate synthesis. The present work is focused on studying the kinetics of the first step from Cetus process, where D-glucose (DG) is oxidized to keto-glucose using P2Ox at optimal pH and oxygenation conditions, with the variation of free enzyme and catalase concentration (0 - 300 U U^{-1} catalase / P2Ox ratio) and for two temperatures (25 ^oC and 30^oC) at which the P2Ox and catalase activity reaches its maximum.

Keywords: D-glucose oxidation; pyranose oxidase; catalase

Abbreviations

ABTS	-	2.2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid
DG	-	D-glucose
DO		dissolved oxygen
GOD	-	glucose oxidase
kDG	-	2-keto-D-glucose
P2Ox	-	pyranose 2-oxidase
U	-	enzyme units (1 unit is the amount of enzyme that catalyses the reaction of 1 µmol of substrate per minute)
U U^{-1}	-	the ratio between enzymatic units of catalase and P2Ox

1. Introduction

The enzymes have become key tools in biotechnology because of their ability to catalytically assist all the transformations of substances in living

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structures, being used both to understand the metabolic pathways, as well as to achieve *in vitro* synthesis of practical interest. The mechanisms that govern such transformations are remarkably precise, being composed of unidirectional cycles performed by coupling – release discrete steps of enzyme, co-enzyme, substrate, intermediates, and products.

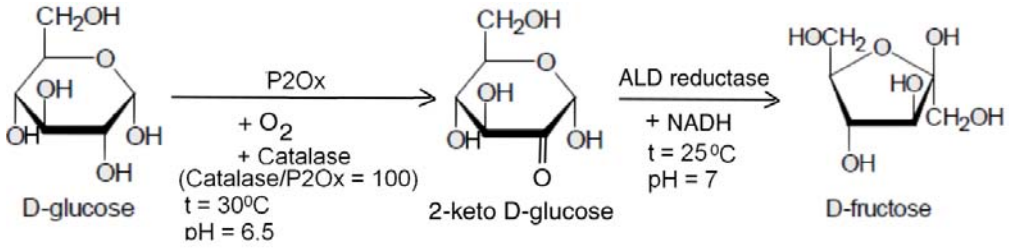
The enzymatic reactions occur with high selectivity and specificity, being valuable alternatives in bioengineering for obtaining a wide variety of food and pharmaceutical products, detergents, derivates for textile industry, or presenting challenging applications in medical-tests, bio-sensor production, or emerging bio-renewable energy industries [1]. Although there are strong similarities between the design and the operation of chemical and biological/biochemical processes, and thousands of enzymes have been investigated in detail, only some dozens are used in significant technical and therapeutic applications [1, 2]. Usually, the enzymes used at industrial scale are those related to the carbohydrate metabolism or hydrolysis of proteins, the most commonly used being amylases from *Bacillus subtilis* (thermal stable), *Aspergillus oryzae* or *Aspergillus niger*, while aminoacylase, streptokinase, catalase, keratinase and phosphodiesterase type I reported a moderate use [3].

The still limited application of enzymes at a large scale can be explained by their high production costs, significant costs of bioprocess industrialization, limited life-time of the enzymes, significant costs of their immobilization (stabilization) on supports, their high sensitivity in relation to the operating conditions, low reproducibility of the bioprocesses (due to the evolving nature of biomass and enzyme characteristics), difficult controllability and a too high substrate specificity.

The use of free enzymes (suspended) in the industrial biosynthesis, in batch or semi-continuous reactors, can be a feasible alternative when the enzymes exhibit a high activity as well as a pronounced deactivation rate, or when the enzyme is cheap compared to the product, and can be easily separated or the product contamination with the enzyme is not important. Alternatively, the use of immobilized enzymes in fixed-bed or fluidized-bed bioreactors has the advantage of an easy separation of the product (with a lower content of allergenic impurities), a smaller loss of enzyme, a higher stability of the enzyme (and possibility of storage and recycling), a higher protection against environmental stress (chemical, physical, mechanical, biological agents), and a better control of the process [4]. However, the immobilization of enzymes is expensive and can lead to a considerable decrease of its activity because of the diffusional transport resistance introduced by the support. In contrast, when the deactivation rate of the enzyme is very high, the use of fixed bed reactors with immobilized enzymes becomes too expensive and inoperable, requiring frequent regeneration / replacement of support-enzyme granules.

Currently, several sugars and sugar-derivates are obtained at an industrial scale either by chemical hydrogenation process with a complex sequence of reactions conducted under severe conditions and with many limitations (high purity of raw materials, extreme temperature and pressure, low yields), or by using more effective enzymatic processes under mild conditions. For example, the enzymatic isomerization of glucose to fructose is thermodynamically limited to a conversion of approx. 50%, while operating at higher temperatures and with recycle requires very costly chromatographic columns for the fructose separation from the 1:1 glucose-fructose syrup. In addition, the used isomerase is less stable and requires a continuous adjustment of the pH and removal of calcium ions existing in the raw materials (solution of glucose) resulting from the starch enzymatic hydrolysis. Purification of fructose from the allergenic aldose is also a problem for this industrial process [1,5,6,7].

In this context, enzymatic oxidation of D-glucose (DG) in the presence of a commercial pyranose 2-oxidase P2Ox (EC 1.1.3.10) is a reaction of high interest in the saccharide industry, due to the possibility to obtain fructose at high yields and free of aldose impurities. The oxidation reaction is the first step of the so-called "Cetus process" [8], D-glucose being oxidized to C2 in the presence of pyranose oxidase (P2Ox) leading to formation of ceto-glucoză (kDG) [9], followed by the kDG reduction to D-fructose [10] according to the following schema:



Because the free P2Ox enzyme is relatively quickly deactivated by some of the oxidation products (H_2O_2, HO•), addition of catalase decomposes the hydrogen peroxide, thus prolonging the POx enzyme activity [10]. The present work is focused on studying the influence of the temperature (in the range of 25-30^{o}C where P2Ox and catalase activity reaches its maximum) and addition of catalase on the efficiency of enzymatic oxidation of D-glucose in the presence of P2Ox under optimal aeration conditions and pH=6.5 (potassium phosphate buffer solution).

2. Pyranose 2-oxidase enzyme

Since the 90s, due to the catalytic properties of P2Ox, the biotechnological interest for this enzyme was growing, due to its use for small / large industrial production of rare sugars or derivatives such as: D-fructose (subsequently biotransformed into D-mannitol), 2-keto-D-gluconic acid (the intermediate in the synthesis of ascorbic acid), 2-deoxy-3-keto-gluconic acid (used for obtaining the 1-deoxy-D-xylulose, i.e. a precursor of thiamine), D-sorbitol, diaminosorbitol, diaminomanitol. [11,12]. Also, P2Ox has found utility in medicine (for quantifying the concentration of 1,5-anhydro-D-glucitol, a very important marker in glycemic control of diabetic patients), in the monitoring of microbial processes or production of biosensors for β-D-glucose [11,13,14,15] The selective reaction is also used to produce several fine chemicals and drugs / antibiotics [16].

In the nature this enzyme is prevalent and can be purified and isolated from several microorganisms including *Phanerochaete chrysosporium* [14], *Phlebiopsis (Peniophora) gigantea* [9,13,17,18]; *Pleurotus ostreatus, Phanerochaete gigantea* [12]; *Polyporus obtusus, Trametes versicolor, Lenzites betulinus, Pleurotus ostreatus, Coriolus hirsutus, Coriolus versicolor, Daedaleopsis styracina, Gloeophyllum sepiarium* [19], *Tricholoma matsutake* [20], etc.

P2Ox is an oxidoreductase which catalyzes the regioselective oxidation reaction of glucose and other sugars to carbon C-2 in the presence of molecular oxygen to form hydrogen peroxide and 2-keto-sugars. Although the catalytic preference of P2Ox for carbohydrate oxidation at C-2 was experimentally proven, recent researches revealed that, in the case of some sugars, the enzyme attacks to carbon C-3 leading to the formation of 3-keto and 2,3-diketo derivates. The reaction mechanism for catalysis with P2Ox is of ping-pong-Bi-Bi type, usually met in the FAD-dependent oxidoreductases.

P2Ox includes four subunits of identical size, and four molecules of flavine-adenosine-dinucleotide (FAD) per tetramer. P2Ox is a large protein with a molecular mass depending on the enzyme source. For instance, P2Ox from *Peniophora gigantea* has a molecular mass of 322 kDa [17], from *Coriolus versicolor* the molecular mass is 290 kDa [21], while those from *Phanerochaete chrysosporium* has 250 kDa (close to 270 kDa P2Ox from *Trametes multicolor,* [14,22], with an approximate diameter of 9 nm for the tetramer. The FAD cofactor is covalently linked to the protein and couples the oxidation of carbohydrates to the subsequent production of H_2O_2 [14].

The activity and stability of the enzyme is strongly influenced by the environmental conditions, respectively temperature and pH. For example, P2Ox from *Phanerochaete chrysosporium* after a week of storage at 4^oC keeps more than 85% of its activity at pH = 4 – 11. Also P2Ox remains 100% active at 60^oC,

65% at 70°C and 35% at 75°C for approximately 2 hours in 50mM Tris-HCl buffer pH = 8 [14]. For P2Ox obtained from *Coriolus versicolor* (isolated and expressed in recombinant *E. coli*) the optimal reaction conditions are: pH = 6 – 8 and 40 – 60°C, the enzyme is stable in the pH range 5 – 9 and at temperature below than 65°C [21,19]. The enzyme isolated from *Tricholoma matsutake* displays an optimal activity at pH 7 – 7.5 and 50 – 55°C, and it is stable at pH > 6 and temperature around the value of 55°C [20]. For P2Ox obtained from *Trametes multicolor,* the optimal reaction conditions are pH 5.5 – 6.5 (dissolved oxygen DO used as oxidant) and 55°C [23]. Depending on the compound used as the electron acceptor, the optimum pH can vary in a wide range from 4 to 8 [23,24].

The stability of P2Ox isolated from *Trametes multicolor* in the absence of substrate is very different from the stability observed under the operating conditions (phosphate buffer, 100mM glucose and 1 U mL^{-1} P2Ox); thus, after 24 hour at 25°C the P2Ox is completely inactivated by the hydrogen peroxide which acts as an oxidizing agent of amino acids (in particular methionine and cysteine). The hydrogen peroxide is stoichiometrically produced during the oxidation of glucose, being difficult to be in-situ removed. Complex enzyme systems, for instance P2Ox / laccase / catalase and another oxidizing agent (quinones) can provide high specific activity of P2Ox (even if oxygen limits the main reaction rate), by maintaining a low concentration of H_2O_2 in the immediate vicinity of the enzyme, thus preventing its rapid consumption [22]. The $OH^{\bullet}$ radicals produced by the decomposition of H_2O_2 have also an important role in the inhibition of the enzyme, because they react almost instantaneously with different amino acids, but especially with tyrosine and methionine. As a consequence, Leitner et al. [23] introduced catalase in the system (isolated from bovine liver) in order to continuously remove the H_2O_2 from the reaction medium by decomposing it into oxygen and water, thus obtaining a complete oxidation of glucose.

3. Experimental section: enzymes, reagents and batch experiments of D-glucose oxidation to kDG

For the present study we used commercial grade compounds from Sigma–Aldrich: recombinant pyranose 2-oxidase (P2Ox, EC 1.1.3.10, product number P4234, specific activity 10.4 U mg-protein^{-1}) from *Coriolus sp.* expressed in *E. Coli*; catalase (EC 1.11.16, product number C1345, specific activity 2860 U mg-protein^{-1}) from bovine liver; glucose oxidase (GOD, EC 1.1.3.4, product number 49180, specific activity 154 U mg-protein^{-1}) from *Aspergillus niger*; peroxidase (EC 1.11.1.7. product number P6782, specific activity 1000 U mg-protein^{-1}) from horseradish; β-D-glucose; 2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS); 0.1 M acetate buffer solution, pH = 5.0; 0.05 M and 0.01 M phosphate buffer solution, pH = 6.5.

Free enzyme experiments have been carried out in a 1 L Sartorius controlled laboratory bioreactor, with the temperature, pH and DO parameters recorded on-line by means of an attached computer [25]. The six-blade disk impeller and the baffles ensure a satisfactory homogenization of the bioreactor content, while the air microsparger system ensures a good gas-liquid contact, the bubble size being dependent on the aeration rate.

The enzymatic oxidation reaction of DG has been conducted in the batch mode at 25°C / 30°C, pH 6.5 with continuous optimal aeration: 1 L min^{-1} rate and 300 rpm stirring speed (values of $k_{oxl}a$ coefficient were 0.005-0.01 s^{-1} for distilled water and 0.01-0.02 s^{-1} for reaction medium, [25]). The assay medium contains 100 mM glucose, 0.25 U mL^{-1} P2Ox, 10 mM phosphate buffer solution and various concentrations of catalase in the range of 0-75 U mL^{-1}. (i.e. 0 - 300 U U^{-1} catalase / P2Ox ratios). The reaction progress was monitored by recording the rate of oxygen consumption by means of an oxygen electrode.

Small samples taken during reaction have been separately analyzed to determine the evolution of glucose, H_2O_2, kDG and active P2Ox concentration (Figs. 1-3). The aliquot samples in vials were immediately stored at -70^{0}C for future analysis of the above mentioned parameters.

Glucose conversion assessment was done using the method of De Luca et al. [26], which relates changes in the UV fluorescence emission spectra with glucose concentration of samples in the presence of free GOD. This approach exploits the UV intrinsic fluorescence of some amino acids from GOD, basically tyrosine and tryptophan [27].

H_2O_2 displays a continuous increase in absorption with the decreasing wavelength in the ultraviolet range and this feature was used for the quantitative determination of hydrogen peroxide by absorption measurement at 240 nm (molar extinction coefficient ε_{240} = 0.00394 ± 0.0002 mM^{-1} mm^{-1}). The difference in absorbance (ΔA_{240}) per time unit is a measure of the catalase activity [28]. The ABTS test of [8] has been done to determine the P2Ox activity during the reaction of interest. This method established that one unit (U) of P2Ox activity is defined as the amount of enzyme necessary for the oxidation of 2 μmols of ABTS per minute under the given conditions.

4. Experimental results

Because P2Ox inactivates quickly under the degradative action of H_2O_2, $HO^{\bullet}$, kDG, and other oxydative species resulted in the reaction environment [9], one alternative is to add catalase in various amounts to decompose H_2O_2, with the beneficial side effect of using the resulted oxygen in the main reaction. However, both P2Ox and catalase present only a limited stability under the mentioned process conditions [29]. Taking into account that the optimum temperature for

free catalase isolated from calf liver is 25°C [30] and for recombinant P2Ox is 35°C, experiments have been carried out at both temperatures (25°C and 30°C), even if from economic reasons the industrial process the recommended temperature is close to the ambient one. Determination of the process kinetics will enable to establish the most favorable operating region in terms of process efficiency that realizes a high DG-conversion and oxidation rate, while keeping a low P2Ox inactivation rate. On the other hand, the study of the process kinetics at two different temperatures is also motivated by multiple and complex interferences of the enzymatic reactions through intermediates that make the activity of the two enzymes to be influenced sometimes in opposite directions. The recorded dynamic evolution of the main species concentrations are displayed in Figs. 1 - 2. Maria et al. [27] have been proposed a complex kinetic model of this process, by accounting for three reactions (Fig. 4): the main oxidation reaction of DG to kDG, the P2Ox inactivation by H_2O_2, and the H_2O_2 decomposition by the catalase. Maria et al. [27] and Ene & Maria [31] have been identified the rate constants for both temperatures of 25°C and 30°C.

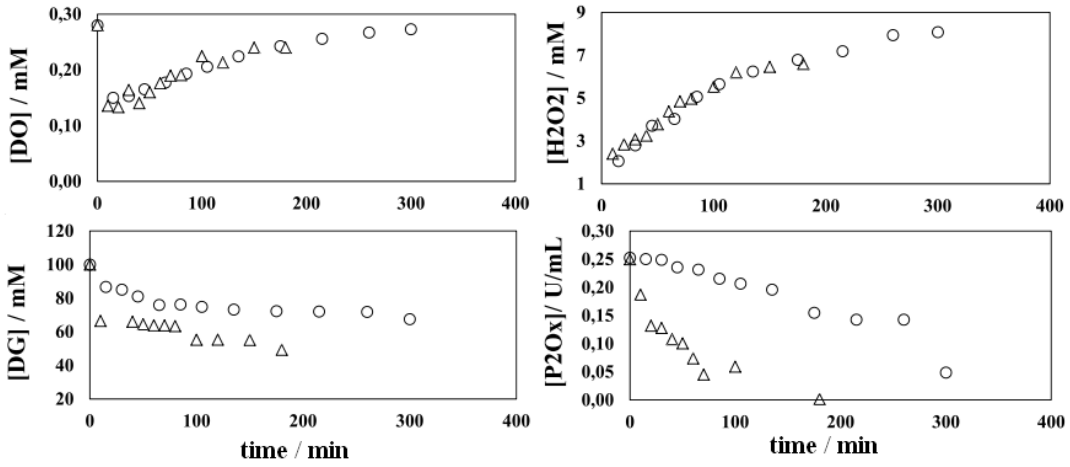


Fig. 1. Evolution of species concentration for D-glucose oxidation with P2Ox in the absence of catalase. Conditions: 100 mM glucose, 0.25 U mL^{-1} P2Ox, 10 mM phosphate buffer, pH = 6.5; o - 25°C; Δ - 30°C.

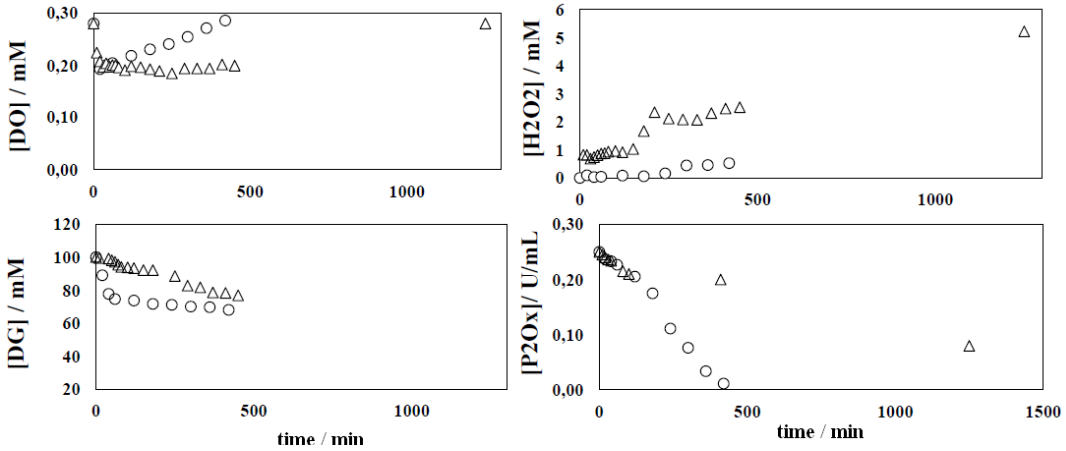


Fig. 2. Evolution of species concentration for D-glucose oxidation with P2Ox in the presence of 25 U mL^{-1} catalase (Catalase/P2Ox = 100/1 U U^{-1}).. Conditions: 100 mM glucose, 0.25 U mL^{-1} P2Ox, 10 mM phosphate buffer, pH = 6.5; o - 25°C; Δ - 30°C

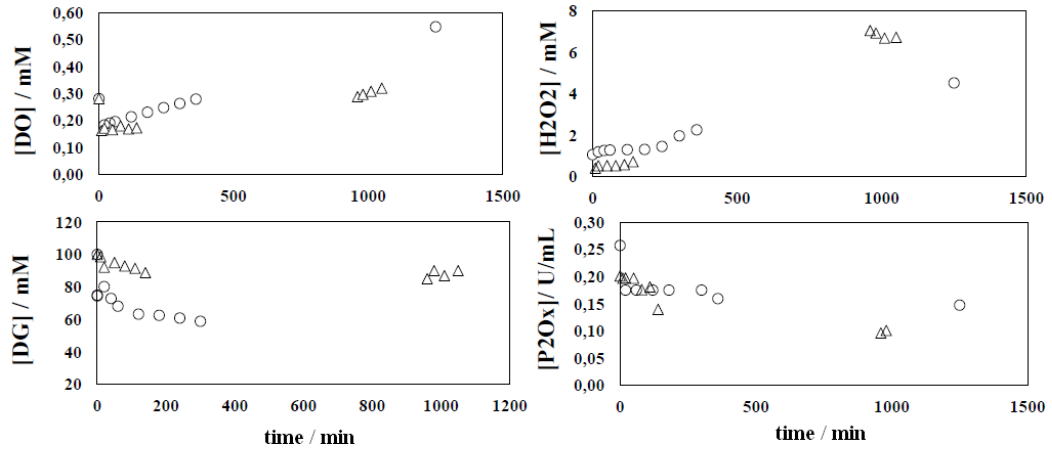


Fig. 3. Evolution of species concentration for D-glucose oxidation with P2Ox in the presence of 80.4 U mL^{-1} catalase (Catalase/P2Ox = 300/1 U U^{-1}). Conditions: 100 mM glucose, 0.25 U mL^{-1} P2Ox, 10 mM phosphate buffer, pH = 6.5; o - 25°C; Δ - 30°C

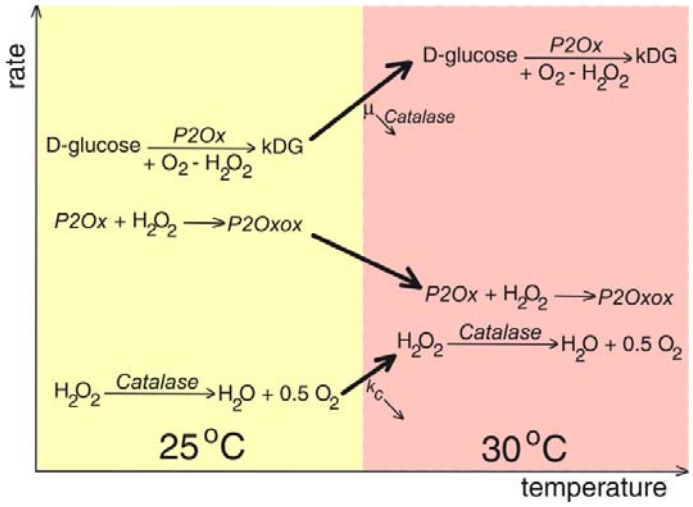


Fig. 4. Influence of temperature in bioconversion of DG into KDG with P2Ox in the presence of catalase, reflected in the main reaction rates considered in the Maria et al. [27] kinetic model

The analysis of the experimental results of enzymatic oxidation of DG with P2Ox at 25°C and 30°C conducts to several conclusions:

i) In the absence of catalase, P2Ox is less active at 25°C than at 30°C (Fig. 1 P2Ox curves). The presence of catalase at 30°C decrease very much the P2Ox inactivation rate, that is ca. 12 times decrease in the inactivation rate constant at 30°C [27], and 39 times in the average deactivation rate for a catalase / P2Ox initial ratio of 300 U U^{-1} vs. no-catalase-runs; (Fig. 3 compared to Fig. 1 P2Ox curves) similar to the data of Leitner et al. [23] at 25°C (P2Ox from *T. multicolor*).

ii) The main reaction of oxidation of DG decreases with increasing Catalase / P2Ox ratio for both temperatures but much faster to 30°C (compare DG-kinetic curves of Fig.1 to Fig. 3, i.e. a 20 times decrease in the maximum rate constant of the extended Michaelis-Menten rate expression; [27]).

iii) The catalase is much active at 25°C than at 30°C, and the H_2O_2 decomposition is faster and results in a low level of H_2O_2 in the reaction environment (below 0.1 mM for 25°C comparatively to avg. 5 mM for 30°C, see H_2O_2 kinetic curves in Fig. 2). Such possible complexes (formed by H_2O_2 with catalase, Fe traces, or by main reaction product/intermediates) might influence the P2Ox inactivation. Indeed, at 25°C where catalase is more active and such complexes are in larger amount (corresponding to low H_2O_2 concentrations), the inactivation of P2Ox is faster in the presence of

catalase. At 30°C, catalase is less active, H_2O_2 concentration is much higher, oxidative complexes are in lower amount, and thus inactivation of P2Ox is lower. For example, if it requires a conversion of 60%, this can be achieved in 3787 min at 25°C for a catalase / P2Ox = 300 U U^{-1} initial ratio and after 1700 min at 30°C using catalase / P2Ox = 100 U U^{-1} (according to the simulations based on the Maria et al. model [27]).

The resulted influence of temperature on the main enzymatic reaction of DG oxidation to kDG is represented schematically in Fig. 4.

5. Conclusion

This study demonstrated, in the experimental basis, the complex interferences between reactions of the bi-enzymatic system of D-glucose oxidation using P2Ox and catalase enzymes (through product / intermediate species). Additional experiments at two temperatures (25°C and 30°C) demonstrate the complex effect of temperature on enzyme activity and intermediate reactions, sometimes in opposite directions. Thus, while the P2Ox activity does not change significantly, the catalase activity reaches its maximum at 25°C by fasting decomposing H_2O_2 to oxidative intermediates leading to a premature P2Ox inactivation.

In contrast, the addition of catalase at 30°C increase the P2Ox life, but a high Catalase/P2Ox ratio (more than 100) leads to a drastic diminution of the main reaction rate.

Acknowledgments

The first author is grateful to Biotehnos Co. (Otopeni, Romania) for the experimental facilities offered with generosity.

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