STUDIES ON THE DYNAMICS OF DEHYDROGENASES KREBS CYCLE ACTIVITY AT *MONILINIA LAXA* (ADERH. & RUHL.) HONEY FUNGUS GROWN ON MEDIA WITH DIFFERENT CARBOHYDRATES

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Abstract: As ubiquitous organisms, fungi grow on a large number of organic substrate, alive or dead, confronting therefore with a wide variety of carbohydrates and various physical factors, and their versatility to adapt and be able to use a large number of these compounds could provide them the chance to survive. Given that, these fungi have a rich enzyme equipment that allows them to operate on different metabolic pathways, this study aims to monitor the dynamics activity of some Krebs cycle dehydrogenases in *Monilinia laxa* (Aderh & Ruhl.) Honey species parasitic on various species of plum trees. To this end, the fungus was cultivated *in vitro* on media enriched with different carbohydrates and the isocitrate dehydrogenase, α-cetoglutarate dehydrogenase, succinate dehydrogenase and malate dehydrogenase activity in the fungus mycelium was followed, at 7, respectively, 14 days after the inoculation of the culture medium and determined using the spectrophotometric Sîsoev and Krasna method (Cojocaru, D.C., 2009). Data revealed obvious differences depending on the type of carbohydrate introduced into the medium and the age of the culture mycelia.

INTRODUCTION

Mitochondria are involved in many essential cellular processes – the production of ATP by oxidative phosphorylation, it participates in various metabolic pathways, contributes to homeostasis and signalling, also plays a key role in apoptosis (Desagher, S. and Martinou, J.C., 2000) and, as the key pivot in adjusting the relative levels of these proteins that make up the type metabolomics systems, and because all the enzymes involved in the Krebs cycle are found in the mitochondrial matrix, these organelles are able to modulate the entire cellular metabolism (Pon, L.A. and Schon, E.A., 2007) but also those of the substrates, forming a dynamic bio-plant that changes its shape and size depending on the cellular and environmental needs. Most active biomolecules such as proteins in a living cell operates as a complex rather than isolated (Beeckmans, S. and Kanarek, L., 1987) these protein-protein interactions having a great relevance for many biological functions. In addition to the most complexes, an increasingly number of such interactions was established, which form complexes rather transitory. Thus, in the metabolomics represented by the tricarboxylic acid cycle at fungi, four enzymes were found-isocitrate dehydrogenase, oxoglutarate dehydrogenase complex, succinate dehydrogenase, malate dehydrogenase, which catalyses the sequential reactions of the Krebs cycle, interacting with aconitase and fumarase and these with other ones that add anaplerotic pathways linked to the biosynthesis of amino acids from intermediate in the Krebs cycle.

All living organisms require energy to maintain their biologic activities. As quantity, the most important elements necessary for the living cells are oxygen, nitrogen, sulphur, phosphorus and especially, carbon. Since it is the basic constituent for all cellular components, it is, therefore, necessary in increased quantities. Unlike green plants capable of using inorganic carbon as CO2, converted into carbohydrates through photosynthesis, non-chloroplastic organisms such as fungi depend entirely of autotrophic organisms for their carbon requirement, and are characterized as chemoautotrophic. Carbon compounds such as carbohydrate are used in the metabolism of fungi for two functions: the first is to supplement the carbon necessary for the synthesis of compounds in the living cell (proteins, nucleic acids, nutritional reserves are included here, among others deriving from the activity and Krebs cycle) and secondary, oxidation of carbon compounds produce appreciable amounts of energy, derivation of the maximum energy from the carbohydrate necessary is complete oxidation undertaken by the tricarboxylic acid cycle enzymes (Gottlieb, D., 1963), fungi may utilize in this regard a wide range of carbon sources, such as: monosaccharides, oligosaccharides, polysaccharides, alcohols, organic acids and lipids (Kadan, M. and Thind, K.S., 1998).

Glucose occupies a key position in fungi metabolism, the degradation of the carbohydrate and various subsequent intermediaries from the energy supply processes of the terminal respiratory processes is quite important for the economy of the cell. Other hexose such as fructose, galactose, mannose, are also considered "metabolic fuels" (Lupea, X.A., 2007). Sucrose and its derived products from its direct catabolism are preferred as source for generating ATP, for the reducing power and for the C skeleton preferred for biosynthetic pathways connected to the Krebs cycle for fungi that belong to different communities. The ability of a compound to donate or receive electrons, described by the redox potential can imprint some difficulties for the substrate during oxidation or reduction, specifically related to a particular relative affinity for electrons that some substances have. For this reason, we can rightly say that there are compounds with a high redox potential such as dextrose cited above, which contains more energy due to the high degree of structural organization, and

other substances, although they may be completely oxidized, they cannot serve as energy sources although they can accept electrons, which influence the cell physiology at different levels (Husson, F. *et al*. 2006).

The amount of carbon source affects profoundly the status of tricarboxylic acid cycle (Poole, R.K., 2010). High positive correlations are found between the known concentration of carbon source and the Krebs cycle intermediate products such as malate, succinate, fumarate and also their negative correlation with the pH of the culture medium (Tarhan, L. *et al*., 2011) and the dependence on hydrocarbon source introduced into the medium.

The ensemble of works that made the experimental design involved determining the enzymes activity that catalyses various Krebs cycle sequences from the mycelium of *Monilinia laxa* (Aderh. & Ruhl) Honey fungus, their quantification and the analysis, on the one hand, of the relationship between the type of carbon source on which the culture medium was supplemented and endoenzyme activity, and, on the other hand, their evolution over time which involved a graphical and statistical approach, and other, from data reported in the scientific literature.

MATERIALS AND METHODS

The *Monilinia laxa* (Aderh. &Ruhl.) Honey inoculum was isolated from mummified fruits from *Prunus domestica* varieties that were collected from the Experimental Orchard of Fruit Research and Development Resort Miroslava, Iasi County. The fungus was cultivated *in vitro* on Leonian medium (in the formula changed by Bonnar), distributed in a quantity of 100 ml in Erlenmeyer bottles and supplemented with 2 g of the following carbohydrates: pentoses such as xylose, hexoses such as dextrose, fructose, disaccharides such as lactose, maltose, sucrose and celobiose, polysaccharides such as soluble starch and glycoproteins, or combinations such as arabic gum and last but not least, polyols such as glycerol, sorbitol and mannitol. We used a control sample devoid of hydrocarbon sources. The culture media were seeded with 8 mm in diameter rings cut from a culture of *Monilinia laxa* (Aderh. & Ruhl.) Honey aged at 7 days and incubated under stationary conditions at 28^0C in the thermostat. The three consecutive experimental measurements which were held at 7 days and 14 days after the inoculation of the culture medium were carried out in fungus mycelium and the Krebs cycle dehydrogenases activity was determined by Sîsoev and Krasna method, modified by Artenie, Vl. (Cojocaru, D.C., 2009). At the basis of this evaluation method of total microbial dehydrogenase activity are these enzymes and their ability to transfer hydrogen from various substrates to 2,3,5 - trifeniltetrazoliu.chloride which reduces to triphenyl-formazan and colours in red, the colour intensity is proportional to the dehydrogenases activity.

RESULTS AND DISCUSSIONS

The isocitrate dehydrogenase is of particular interest because it controls the carbon flux between the Krebs cycle and bypasses glyoxylate by kinase / phosphatase isocitrate dehydrogenase activation and inactivation (Laporte, D.C, and Koshland, D.E.J. 1982, Laporte, D. C. *et al*, 1985). Thus, activation of the ongoing forces into the Krebs cycle causes a decrease of isocitrate at cellular level and an increase of α-ketoglutarate levels (O'Roy, S. and Packard, T.T., 1998). The concentration of intracellular intermediate metabolites and thus, the enzyme level is dependent on time, the growth phases of submerged culture, and of NADH concentration throughout these phases. The pyruvate is also known as an activator for IDH phosphatase, an enzyme responsible for activating isocitrate dehydrogenase (Gálvez, S., and P. Gadal., 1995). In some microbial cultures with different carbon sources, isocitrate dehydrogenase, the enzyme that reflects the increased respiratory rate, increased in the early exponential growth phase and the plateau phase of culture, but also at the end of this. Also, the same data indicates that depleting the culture hydrocarbon sources is followed by a full depression of isocitrate dehydrogenase activity, to fill other cellular needs, which are maintained at lower levels, but in a state of alert (Roy, S.O. *et al*., 1999).

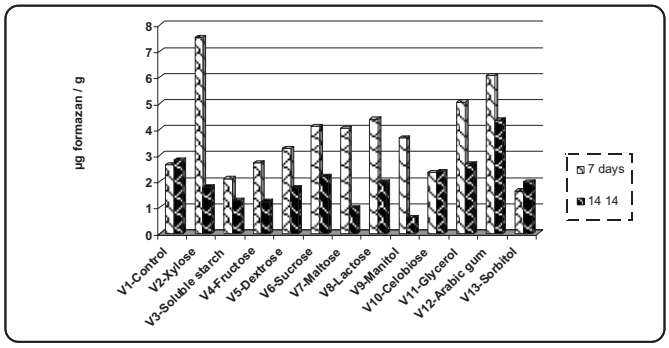

Fig. 1. The influence of carbohydrates on the dynamics of isocitrate dehydrogenase activity at *Monilinia laxa* (Aderh. & Ruhl.) Honey fungus

At a rigorous analysis of the enzyme activity dynamics that catalyses the first step of the Krebs cycle as it is depicted in Figure 1, we found that all medium variants supplemented with carbohydrates, decreased with the aging of mycelia culture, except the witness, where an increase in the activity was observed, that rose to 2.6520 µg formazan / g.mat. to 2.8172 µg formazan / g.mat. In the medium sample enriched with sorbitol the activity of isocitrate dehydrogenase amplified from 1.6255 µg formazan / g.mat. to 1.9709 µg formazan / g.mat., and in the sample were celobiose was added, a tiny increase appeared, from 2.3437 µg formazan / g.mat. to 2.3739 µg formazan / g.mat. Thus, the strong fluctuations of the enzyme in the mycelium were recorded for the following carbohydrates: xylose - from 7.5226 µg formazan / g.mat. to 1.7731 µg formazan / g.mat., maltose - from 4.0352 µg formazan / g.mat to 0.9930 µg formazan / g.mat.and manitol from 3.6551 µg formazan / g.mat. to 0, 6219 µg formazan / g.mat followed by one filled with lactose - from 4.3881 µg formazan / g.mat la 1.9768 µg formazan / g.mat and with glycerol at the 5.0454 µg formazan / g.mat.to 2.6769 µg formazan / g.mat while in the medium enriched with sucrose, the enzyme presented a variation from 4.1032 µg formazan / g.mat.to 2.2096 µg formazan / g.mat, in that with arabic gum resulted a fluctuation from 6.0718 µg formazan / g.mat. to 4.3493 µg formazan / g.mat Dextrose had a similar behaviour in the mycelium on isocitrate dehydrogenase, reducing its activity from 3.2663 µg formazan / g.mat to 1.7503 µg formazan / g.mat. Inhibitory effects between the two time intervals had also fructose, the activity of the endoenzyme decreasing from 2.7209 µg formazan / g.mat. to 1.2243 µg formazan / g.mat but starch determinate a decline in the action of the enzyme that declined from 2.1007 µg formazan / g.mat. to 1.2809 µg formazan / g.mat.

The carbohydrates metabolism via tricarboxylic acid cycle involves the participation of α-ketoglutarate multienzyme complex, that consists of the following enzyme subunit: a ketoglutarate dehydrogenase (E1), dihydrolipoyl transacetylase (E2) and dihydrolpoyl dehydrogenase (E3), the complex being responsible for the oxidative decarboxylation of α-ketoglutarate by the intervention of coenzyme A to succinyl ~coenzime A.

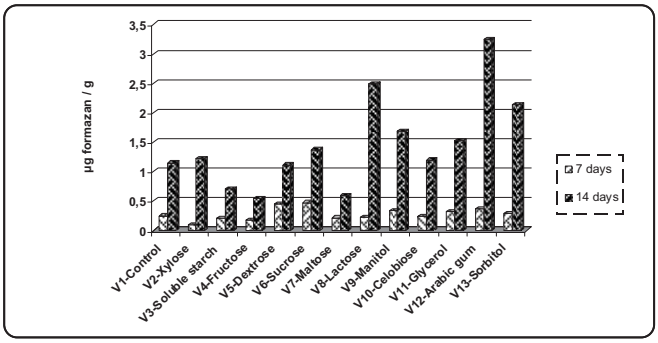

Fig. 2. The influence of carbohydrates on the dynamics of α-ketoglutarate dehydrogenase activity at *Monilinia laxa* (Aderh. & Ruhl.) Honey fungus

The results regarding the activity of oxoglutarate dehydrogenase in the mycelium of *Monilinia laxa* fungus grown on media enriched with different carbon sources are graphically shown in Figure 2. You can see clear differences in the overall activity of the enzyme, suggesting a specific metabolic activity depending on the substrate available for the fungus but also the fact that age of the culture affects both nutrition, respiration and microbial dynamics.

The dynamic profile of this biologically active molecule that catalyses the decarboxylation of α-ketoglutarate is characterized, after careful analysis, by an upward increase in the biosynthesis and enzyme activation, in direct proportion to the aging of the culture of *Monilinia laxa* fungus for all carbohydrates, including the control sample. For the vast majority of medium variations, the increased enzyme activity had the most intense growth found in the variant with gum arabic: from 0.3707 µg formazan / g.mat. to 3.2507 µg formazan / g.mat. In the case of lactose, from 0.2228 µg formazan / g.mat. it reached a peak of 2.4919 µg formazan / g.mat, while sorbitol stimulated the enzyme activity in time from 0.2842 µg formazan / g.mat to 2.1339 µg formazan / g.mat and mannitol from 0.3380 µg formazan / g.mat to 1.6850 µg formazan / g.mat. Glycerol proved to be also a good nutrient, increasing in time the performance of α ketoglutarate dehidrogenase activity that climbed from 0.3219 µg formazan / g.mat to 1.5195 µg formazan / g.mat. and sucrose induced an similar behaviour to the enzyme, its variation going from 0.4696 µg formazan / g.mat to 1.3720 µg formazan / g.mat. Same happened with xylose - from 0.0925 µg formazan / g.mat to 1.2126 µg formazan / g.mat. In the case of the medium with celobiose, oxoglutarate dehydrogenase increased in time, from 0.2367 µg formazan / g.mat to 1.1948 µg formazan / g.mat and in the variant supplemented with dextrose, from 0.4505 µg formazan / g.mat to 1.1159 µg formazan / g.mat. Starch caused a more modest time activity for the endoenzyme, varying from 0.2086 µg formazan / g.mat. to 0.6985 µg formazan / g.mat., while maltose, fructose, determined a similar behaviour as starch, α-ketoglutarate dehidrogenase

activity changed from 0.2133 µg formazan / g.mat. to 1.1159 µg formazan / g.mat, respectively, from 0.1671 µg formazan / g.mat to 0.5401 µg formazan / g.mat.

Because the Krebs cycle enzymes, like those of hexozo-monophosphate shunt and Emden-Meyerhoff-Parnas pathway are respiratory enzymes, the increased oxoglutarate dehydrogenase activity at *Monilinia laxa* suggested in the second interval, an increase in respiratory rate, dependent of the age of the fungus and an intense activity of the enzymes from the antioxidant defense line, knowing that a high metabolic rate is followed by the increase of oxidative stress markers that are responsible for the aging of mitochondria, which are the main source of ROS (Tahar, E.B. *et al*., 2007) due to multiple reactions that transfer electrons. In the electron transport chain, a small amount of electrons are distracted by oxygen to intermediate points, such as complexes I and III that generate superoxide anion radicals, which are converted inside the mitochondria in H_2O and other species of ROS (Kowaltowski , A.J., and Vercesi, A.E. 1999). In addition to the electron transport chain, recent works showed that enzymes soluble in the mitochondrial matrix as pyruvate dehydrogenase and α-ketoglutarate dehidogenase can also generated species of ROS, being the primary site for this (Starkov, A.A. *et al*, 2004), not just a target for oxidative stress. As each source of mitochondrial ROS respond differently to substrates, changes in the energy metabolism, O_2 and tensions (Turrens, J.F., 2003), as a result, each generation mitochondrial apparently, in this study works to lift parameters, regardless the age but with emphasis on the mature one.

Succinate dehydrogenation is catalysed by succinate dehydrogenase, a flavoprotein which, in eukaryotes, is the only enzyme of the citric cycle trapped deep in the internal mitochondrial membrane (Garret, R. and Grisham, C.M., 2005). The enzyme contains succinate dehydrogenase complex, the only complex of the respiratory chain that pumps protons, although it has a transmembrane domain that introduces electrons taken from $FADH_2$ into the respiratory chain whose energy is too high, surrendering CoQ to electron carriers of the respiratory chain (Cecchini, G. 2003). Examination of the course of activity of the enzyme that is converting succinate to fumarate, in time, signals not only an increase in the oxidative stress due to production of reactive oxygen species (ROS), expression of a strong aerobic metabolism in the medium variations, but could reflect a change in the fraction of the active enzyme, due to change of metabolic conditions and an intensification of the respiratory process, which removes the idea that at 4 days after medium inoculation, the mycelium of *Monilinia laxa* fungus is old, since that some data from the literature indicates that this ubiquinone activity decreases with age and nutrient depletion in the culture medium but also because some cellular components are known to be sensitive to oxidative stress, specifically proteins containing Fe-S clusters of succinate dehydrogenase as well, highly sensitive to superoxides resulting from the metabolic activity (Masoro, E.J. and Austad, S.N., 2006). There have been variations of the succinate dehydrogenase activity (graphically seen in Figure 3), that increased from 0.1373 µg formazan / g.mat to 1.3414 µg formazan / g.mat. For version control, to provide a modest increase in medium supplemented with fructose - from 0.2413 µg formazan / g.mat to 0.3360 µg formazan / g.mat. and to have a powerful amplification in the mediums enriched with arabic gum from 0.3785 µg formazan / g.mat to 2.3695 µg formazan / g.mat with sorbitol- from 0.0778 µg formazan / g.mat. to 1.1880 µg formazan / g.mat. and mannitol from 0.2516 µg formazan / g.mat. to 1.3578 µg formazan / g.mat. Succinate dehydrogenase had a similar behaviour in the mediums with lactose supplement, where it increased from 0.1327 µg formazan / g.mat. up to 2.1652 µg formazan / g.mat. and with celobiose where it jumped from 0.0050 µg formazan / g.mat. to 0.6536 µg formazan / g.mat.

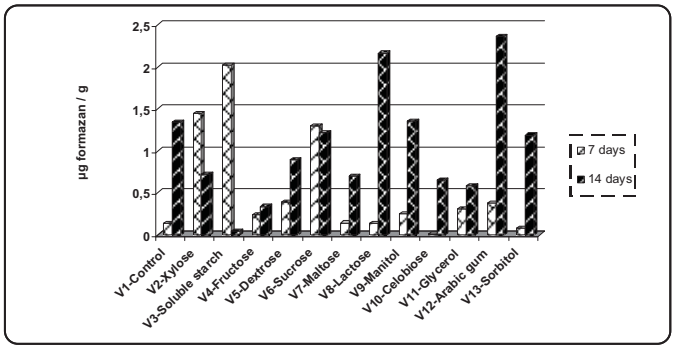

Fig. 3. The influence of carbohydrates on the dynamics of succinate dehydrogenase activity at *Monilinia laxa* (Aderh. & Ruhl.) Honey fungus

The addition of maltose was followed by an increased activity of the endoenzyme from 0.1414 µg formazan / g.mat.. to 0.6967 µg formazan / g.mat. while dextrose (V5) induced an increase of biosynthesis and the enzyme action in time, rised from 0.3857 µg formazan / g.mat. to 0.8900 µg formazan / g.mat.., and glycerin stimulated the enzyme activity from 0.3062 µg formazan / g.mat. to 0.5804 µg formazan / g.mat. Filling the basal medium with sucrose was not followed

by a significant variation of the enzyme specific for the third stage of the Krebs cycle, this had a tiny decrease, from 1.2940 µg formazan / g.mat. to 1.2171 µg formazan / g.mat. More intensive declined succinate dehydrogenase activity levels over time in medium variations with added starch, which decreased from 2.0177 µg formazan / g.mat. to 0.0400 µg formazan / g.mat.. and with addition of xylose, which decreased from 1.4476 µg formazan / g.mat.. to 0.7232 µg formazan / g.mat.

Both maturation and aging of submerged fungal culture in medium with different carbon sources can be followed by the accumulation of toxic metabolites. Typically, the cellular respiration coupled with ATP synthesis decreases during aging, α- ketoglutarate dehydrogenase and malate dehydrogenase decreasing significantly, while the of activity isocitrate dehydrogenase decreases more and is completely inhibited in the oldest culture. By contrast, the literature emphasizes that succinate dehydrogenase is more active. The behaviour of oxidative enzymes and of metabolic pathways are apparently inherent in long-lived cells from the population, selected by genetic fitness during chronological aging (Samokhvalov, V. *et al.,* 2004), depending on genetic background as well as epigenetic regulation through interaction with environmental factors.

The last stage in the Krebs cycle, in which L malate is oxidize to oxaloacetate is catalysed by the malate dehydrogenase. The graphical representation of the enzyme activity as it appears in Figure 4 illustrates a different dynamic to others oxidoreductases from the Krebs cycle, but relatively similar to isocitrate dehydrogenase in the variation curve. The careful examination of malate dehydrogenase activity dynamics helps us to see that there is a tendency to decrease in the of activity malate dehydrogenase in the second time period for the experimental measurements, confirming the data from literature, that show that with the maturation and aging of the microbial cultures, malate dehydrogenase activity decreases.

So, it can be seen that the culture medium with arabic gum, endoenzyme activity remained almost constant, with an infinitesimal variation in the negative direction, down from 3.4901 µg formazan / g.mat to 3.4006 µg formazan / g.mat, while the presence of sorbitol in the medium induced an increase in its activity, while it increased from 1.7935 µg formazan / g.mat to 2.5088 µg formazan / g.mat. The remaining curves of variation regardless the carbon source of glucidic nature introduced in the culture medium, have a descending path. The most powerful decrease was the variant with xylose, the enzyme descending from 5.4696 µg formazan / g.mat. at 7 days after inoculation up to 1.4594 µg formazan / g.mat. in the second period. V8 version (with lactose) showed a degressive variation, moving from 4, 2624 µg formazan / g.mat. at a threshold of 1.0293 µg formazan / g.mat., while the version with glycerol (V11) malate dehydrogenase activity recorded a decrease from 4.2025 µg formazan / g.mat.la 2.2820 µg formazan / g.mat., followed by the sucrose version (V6) of 3.5788 µg formazan / g.mat. to 1.9801 µg formazan / g.mat. and the culture with the addition of mannitol (V9), where values have dropped from 3.2707 µg formazan / g.mat. to 0.8937 µg formazan / g.mat.

Starch induced a decreasing malate dehydrogenase activity from 2.6387 µg formazan / g.mat. to 1.4566 µg formazan / g.mat.., while the control sample, without carbohydrates had activity levels formazan 2.5415 µg formazan / g.mat.at 7 days, respectively, 1.7822 µg formazan / g.mat. at 14 days. The mycelium of *Monilinia laxa* grown on the medium enriched with glucose showed a decreased malate dehydrogenase activity, from 2.4179 µg formazan / g.mat.to 2.0069 µg formazan / g.mat.

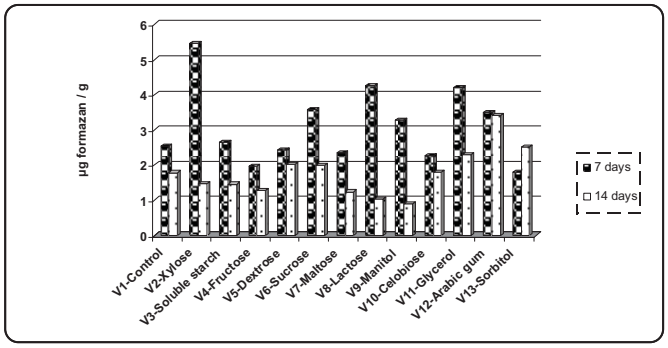

Fig. 4. The influence of carbohydrates on the dynamics of malate dehydrogenase activity at *Monilinia laxa* (Aderh. & Ruhl.) Honey fungus

This enzyme behaviour demonstrates that glucose kept an approximately constant redox potential, the amount of energy released is significantly undiminished through time. With regard to maltose it induced a reduction of malate dehydrogenase activity in the fungus mycelium, while it halved its energy resources that enter in the Krebs cycle from 2.3504 µg formazan / g.mat. to 1. µg formazan / g.mat. Endoenzyme values found under the influence of celobiose also

decreased from 2.2665 μg formazan / g.mat. to 1.8023 μg formazan / g.mat. while, under the action of fructose (V4 version), values went from 1.9563 μg formazan / g.mat to 1.2680 μg formazan / g.mat.

CONCLUSIONS

After 7 days of incubation, the isocitrate dehydrogenase activity was stimulated in media supplemented with xylose, arabic gum, glycerol, lactose, sucrose, maltose, mannitol, dextrose and fructose, and after 14 days, only in the medium with arabic gum.

The α-ketoglutarat dehydrogenase biosynthesis was stimulated in the young mycelium by sucrose, dextrose, arabic gum, mannitol, glycerol and sorbitol, and in the aged culture by arabic gum, lactose, sorbitol, mannitol, glycerol, sucrose, xylose and celobiosys

The succinate dehydrogenase activity was stimulated at 7 days after sowing by starch, xylose, sucrose, dextrose, arabic gum, glycerin, mannitol, fructose, maltose, and at 14 days by arabic,gum lactose and mannitol.

In the 7 days old mycelium, the malate dehydrogenase was stimulated by xylose, lactose, glycerol, sucrose, arabic gum, mannitol and starch, and in the 14 days old mycelium by arabic gum, sorbitol, glycerin, dextrose, sucrose and celobiozã

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