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Improved Production of α-Amylase by *Aspergillus terreus* in Presence of Oxygen-Vector

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Abstract: *n*-Dodecane has been investigated as an oxygen-vector for improving α-amylase biosynthesis using the strain *Aspergillus terreus*. In aerobic microbial cultivation, continuous supply of oxygen is required especially due to its low solubility in the growth medium, in particular at high viscosity, but the limitations of oxygen mass transfer in these systems can be overcome by the addition of water-insoluble compounds which possess a strong affinity for oxygen, namely oxygen-vectors. The use of *n*-dodecane (as an oxygen-vector) in the fermentation medium of *A. terreus* can significantly improve the bioprocess performance and enhance α-amylase production. Using 5% *n*-dodecane at 35 °C, an increase of 1.8–2 times in the enzymatic activity was recorded. In the oxygen-vector's absence, the highest amount of biomass was obtained at 35 °C, while in the presence of 5% vol. *n*-dodecane, the amount of fungal biomass increased by approximately 70%, with a shift in optimum temperature to 40 °C, generating also an enzymatic activity increase of 2.30 times. Moreover, the oxygen-vector's addition in the fermentation broth influenced the fungal morphological development in the form of larger pellets with a more compact structure compared to the system without *n*-dodecane, with a positive effect on the fermentation performance (higher α-amylase activity production).

Keywords: *Aspergillus terreus*; α-amylase; enzymatic activity; oxygen-vector

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1. Introduction

Amylases are one of the most important hydrolytic enzymes, in terms of their use at the domestic or industrial level, fungal amylase being the first enzyme produced at an industrial scale in 1894, using the strain *Rhizopus oryzae* [1].

α-Amylases catalyze the cleavage of α-1,4-glycosidic bonds from starch with the formation of lower molecular weight compounds, such as glucose, maltose, and oligosaccharides (maltotriose, dextrin) [2]. Although microbial, plant, or animal sources can be used to obtain α-amylases, the most utilized are microbial α-amylases (of bacterial, yeast, or filamentous fungal origin) due to the wider versatility in industrial applications (food, bakery, beverages, textiles, detergents, as well as pharmaceutical, clinical, or analytical uses) [2]. In fact, the chemical process for obtaining glucose by starch hydrolysis has been replaced almost completely, at the industrial level, by the enzymatic technology. In Asia, α-amylases, mainly fungal, are widely used to make traditional fermented foods, such as sake, sweet sake (koji amazake), soy sauce, and miso soup [3].

Due to its multiple applications, α-amylase is included in the category of "industrial enzymes", along with proteases and lipases, their market being in continuous growth, mainly due to biotechnology advances (in 2020, the market of industrial enzymes was estimated at over $6 billion, of which about 25% corresponded to amylases) [4].

In order to obtain α-amylases, microbial cultures are preferred because microorganisms can easily grow in large quantities and, implicitly, can ensure high enzyme productions.

The biosynthesis of α-amylases is performed in submerged or in solid state fermentations, the second case being specific to fungal cultures, as they are more naturally adapted to grow in a medium with low free water [5]. Enzymes produced by fungi have the advantage that they are extracellular products, which simplifies the fermentation broth downstream processing. In addition, fungi can grow in a variety of environments, even solids, and the biosynthesized α-amylases are preferred to those from other microbial cultures due to their GRAS (Generally Recognized as Safe) classification by the FDA (United States Food and Drug Administration) [6].

Thus, for α-amylase production, *Aspergillus* sp., *Penicillium* sp., *Rhizopus* sp., *Mucor* sp., *Thermomyces* sp., *Thermonospora* sp. [7,8] are used, which have the ability to grow on noncomplex culture media, generally by-products: wastewater from vegetable processing, banana peels, rice husks, molasses, wheat bran, straw, corn cobs and leaves, oilseed cakes, etc. [9].

Fermentation processes' optimization in order to obtain α-amylase must take into account many factors, including pH, temperature, carbon and nitrogen sources, the presence of metal ions in the environment (calcium, cobalt, magnesium, sodium, manganese), phosphate ions, etc. [8,10]. In addition, in submerged fermentations, mixing and aeration play important roles in achieving high productivity. Both aeration and agitation systems have a higher influence in fungal fermentations, mainly due to their determined role on the microorganisms' morphology and, implicitly, on the nutrients' consumption rates and enzyme biosynthesis [11].

Efficient aeration involves intense mixing or high air flow rates, both of which can generate mechanical forces that would destroy microbial cells. For this reason, a variety of oxygen-vectors (n-alkanes: *n*-dodecane, n-hexadecane; perfluorocarbons; vegetable oils) can be used to ensure in the fermentation broth an efficient oxygen supply in mild mechanical or pneumatic mixing conditions [12]. Oxygen-vectors are organic water-insoluble compounds, which have the ability significantly to increase the concentration of dissolved oxygen in the fermentation broth and its transfer rate from the gas phase to the microorganisms without the need to change the mixing or aeration conditions [13,14]. Obviously, oxygen-vectors should not be toxic to microorganisms, as they are often additional sources of nutrients. The addition of these compounds to the aerobic cultures of several microorganisms, through the effect induced on the oxygen mass transfer, can lead to an increase in target compound productivity [12,15]. Lai et al., 2002 investigated the use of *n*-dodecane in *A. terreus* cultivation for lovastatin production, registering an increase of 1.4 fold for 2.5% oxygen vector added in the media [16]. Zhang et al., 2018 reported an increase in fumarase activity to 124% for 2.5% *n*-dodecane used as the oxygen vector in the biosynthetic process using recombinant *E. coli* BL21-pET22b-fumR, without significant changes in the expression of the enzyme [17]. Xu et al., 2020 analyzed several oxygen vectors for the biosynthesis of L-amino acid oxidase and obtained an increase in enzyme activity of 8.6% for *Bacillus subtilis* HLZ-68 using 1.5% *n*-dodecane [18]. Amaral et al., 2008 studied the addition of 20% perfluorodecalin in a bioreactor with YPD (Yeast Extract–Peptone–Dextrose) medium, and 230% enhancement was registered [19]

In this context, previous studies on the positive effects of oxygen-vectors on aerobic fermentation processes are continued by analyzing the influence of *n*-dodecane on the biosynthesis of α-amylase using the fungus *A. terreus*. In this purpose, the effects of *n*-dodecane presence in the environment on the morphological characteristics of the fungi and, through morphology, on the enzyme production in the fermentation broth, were investigated. The information obtained is integrated into a broader framework with a number of other parameters, such as oxygen-vector concentration, biomass concentration, and fermentation broth viscosity.

2. Materials and Methods

2.1. Bioreactor and Operating Parameters

The fermentations were carried out in a batch system, using, in this purpose, the laboratory stirred bioreactor Fermac (1 L working-volume). The bioreactor was provided with one impeller of turbine type and three baffles. The bioreactor geometrical characteristics were previously given [12]. The fermentations were carried out at 35 °C (in the experiments studying the temperature influence, the fermentation temperature was 30, 35, or 40 °C). Depending on the experimental program, the pH-value was either allowed to vary freely, or maintained at 6 using a sterilized solution of 0.1 M NaOH. The air was distributed inside the broth through a perforated tube of 7 mm diameter, placed at 15 mm from the bioreactor bottom. The sparger was provided with 4 holes of 1 mm diameter. The air volumetric flow rate was 5 L/min, and the rotation speed of the impeller was maintained at 150 rpm.

2.2. Strain and Medium

The fungus *A. terreus* ATCC 32588 was used in the experiments. Before the fermentation process, the inoculum medium was prepared: sucrose 1.5 g, $NaNO_3$ 0.1 g, $MgSO_4$ 0.025 g, KCl 0.025 g, $FeSO_4$ 0.0005 g, K_2HPO_4 0.05 g for 50 mL [20]. The spores were germinated and the fungal cells grown at 35 °C in an incubator-shaker, at 150 rpm, for one day. The fungal biomass was transferred into the bioreactor, which contained a specific medium: soluble starch 6.7 g/L, 0.47 g KH_2PO_4, NH_4NO_3 3.3 g/L, KCl 0.16 g/L, $MgSO_4$ 0.03 g/L, $CaCl_2$ 0.13 g/L, $FeSO_4$ 0.003 g/L [20]. For controlling the foam formation and level, the silicone-based antifoam agent was selected (Antifoam 204, Merck, Darmstadt, Germany). The inoculum was added at 10% vol. after the sterilization of this medium at 121 °C for 20 min in a RAYPA AES-28 autoclave. Sterilized *n*-dodecane was used as the oxygen-vector, with specific characteristics related to its utilization for enhancing the oxygen transfer rate: density 750 g/L at 20 °C, oxygen solubility $54.9 \cdot 10^{-3}$ g/L at 35 °C, and atmospheric air pressure [12]. The hydrocarbon experimented concentrations into the broth varied between 2.5 and 10% vol.

2.3. Measurement and Analysis Methods

The biomass accumulation was analyzed by centrifugation of collected samples, drying at 80 °C until constant weight was achieved in order to calculate microbial biomass (d.w.) expressed in g/L. The supernatant, separated from the *n*-dodecane by centrifugation at 5000 rpm, was used for amylase analysis. The pellets' diameter was analyzed using an Optika Microscope B380 (Ponteranica, Italy) equipped with an Optika CB-10 video camera (Ponteranica, Italy) and Optika PROView software (Ponteranica, Italy). From each sample, 10 pellets were separated and evaluated for shape and diameter.

The activity of α-amylase was measured during the fermentation cycle using a commercial kit Amilase 405, kinetic unitest (Winer Lab., Rosario, Argentina). The analysis consists of measuring the absorbance at 405 nm of the colored 2-chloro-*p*-nitrophenol released from 2-chloro-*p*-nitrophenyl-α-D-maltotriose under enzyme action [21]. The enzymatic reaction occurred at 25 °C, in phosphate buffer medium, the optimum pH value being 6.00. The unit of enzyme activity was calculated as the amount of enzyme required to hydrolyze 1 μmole of substrate per minute [21]. The values of the oxygen transfer rate, quantified by means of k_La, were calculated using the dynamic method.

Each experiment was repeated three times, using identical conditions, the average value of measured parameters being used in calculations. The maximum error varied between 5.66 and 7.23%.

3. Results

3.1. The Oxygen-Vector Effect on the Fermentation Broth pH

Fungi are producers of carboxylic acids, either as the major product or as by-products (*Aspergillus* sp. Produces citric, gluconic, malic, and itaconic acids). Moreover, under stress conditions, such as limiting oxygen or nutrients, fungi can alter their metabolism, increasing

the production of carboxylic acids [22]. For these reasons, without strict control, the pH decreases during fermentation.

In this context, according to Figure 1, the pH evolution during the fermentation of *A. terreus* is different in the two systems, depending on oxygen-vector presence: in the first two days, the pH value is similar in both fermentation processes, with and without *n*-dodecane. The differences appear from the third day, the pH of the environment without the oxygen-vector decreasing faster. As fermentation progresses, these differences become more pronounced, especially as the pH value for the system containing the oxygen-vector varies very little from the fourth day. As previously mentioned, in the Introduction Section, in the absence of the oxygen-vector, the level of oxygen concentration is lower [13,14], and the destruction of pellets with formation of the filamentous mycelium further reduces the oxygen and nutrients transfer rate [22].

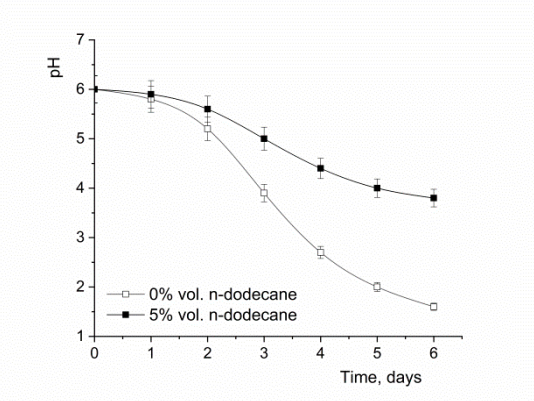


Figure 1. Comparison between the pH-values during the fermentation in absence and presence of *n*-dodecane.

Under these conditions, the metabolic response of *A. terreus* strains materializes mainly in the itaconic acid biosynthesis [23,24]. The acid accumulation as the main product of biosynthesis significantly reduces the pH value compared to the fermentation in the presence of *n*-dodecane.

3.2. The Effect of the Oxygen-Vector on A. terreus Morphology

It is known that fungi can develop in two morphological forms: dispersed mycelia and compact pellets. The fungal morphology is determined both by the genetic information and by the cultivation conditions in the bioreactor (dissolved oxygen level, pH value, mixing intensity, etc.). The strains of *A. terreus* used in these experiments grow under normal conditions in the form of pellets [25]. In the case of α-amylase biosynthesis, important differences in the morphological structure of the fungi were found in the absence or presence of *n*-dodecane. A first aspect observed is that by adding the oxygen-vector, the size of the formed pellets increases (Figure 2).

Moreover, *n*-dodecane supports the maintenance of the pellets' structural integrity throughout the fermentation process; only after the fifth day was destruction of pellets noted, as a consequence of nutrients' depletion in the environment. In the absence of the oxygen-vector, as the process progresses and, implicitly, as the biomass accumulates, branched forms of *A. terreus* appear, with the degradation of the spherical shape of the pellets from the second day of fermentation (Figure 3).

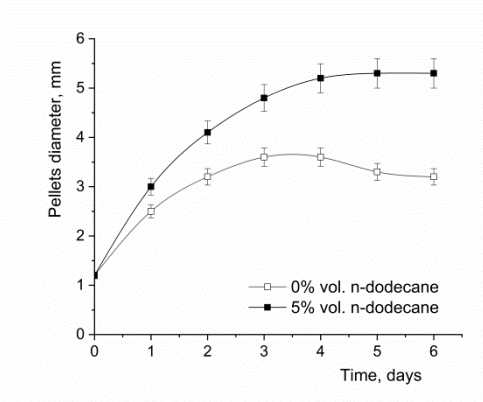
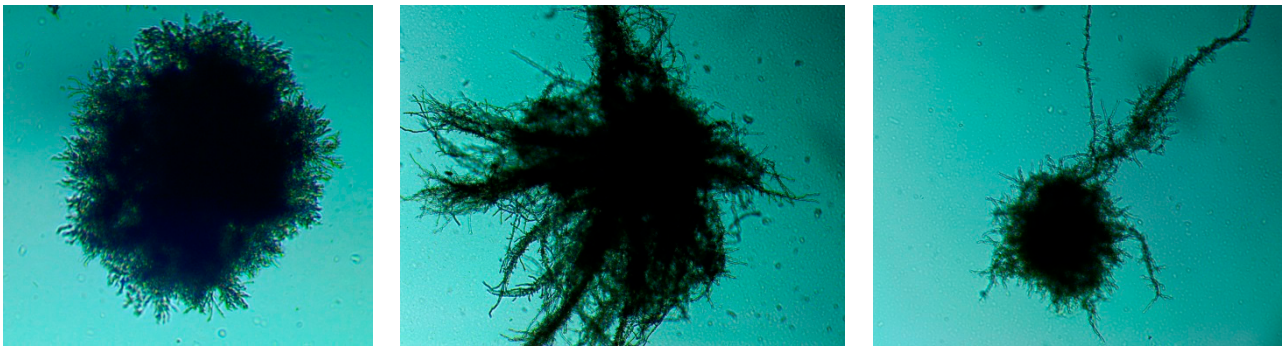


Figure 2. Comparison between the pellets' size in absence and presence of *n*-dodecane.

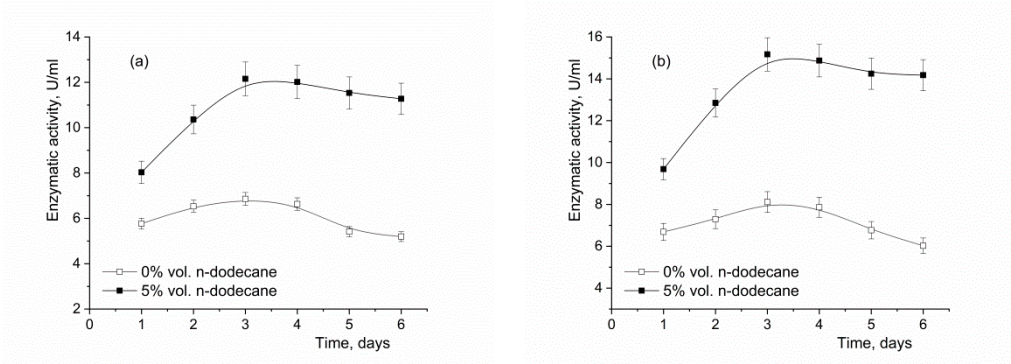


(**a**) (**b**) (**c**)

Figure 3. Pellets' shapes in presence of *n*-dodecane (**a**) and absence of *n*-dodecane (**b**,**c**) on the third day of fermentation.

3.3. The Effect of the Oxygen-Vector on the Biomass Accumulation and α-Amylase Activity

The oxygen-vector beneficial impact is potentiated by maintaining the pH value constant at 6 (Figure 4). From this figure, it can be noted that, regardless of the system used, with or without *n*-dodecane, maintaining the pH at 6 generates higher enzyme activities compared to the fermentation process in which no pH control is applied, the increase being about 1.20–1.40 times; the addition of *n*-dodecane could partially compensate the uncontrolled variation in pH during fermentation.



(**a**) (**b**)

Figure 4. Variation of α-amylase activity during the fermentation cycle for uncontrolled pH-value (**a**) and adjusted pH-value at 6 (**b**).

At the same time, for both used systems, with or without pH regulation, from Figure 4, it can be noted that the addition of *n*-dodecane generates a significant increase in enzyme

production, about 1.8–2.0 times. In both fermentation systems, the maximum activity of the enzyme is reached on the third day after the beginning of the process. However, according to Figure 5, increasing the volume fraction of the oxygen-vector may have a negative effect on α-amylase activity. Thus, as the oxygen-vector concentration increases, the enzymatic activity increases, reaches its maximum, after which it decreases. The variation is the result of two opposite effects that occur with increasing *n*-dodecane content in the fermentation broth: on one side—the increase in the amount of dissolved oxygen and its transfer rate, and on other side, as consequence of the former effect—the appearance of the oxygen inhibitory effect, known as oxidative stress [12,24]. Under these circumstances, Figure 5 suggests that the optimum concentration of *n*-dodecane is 5% vol.

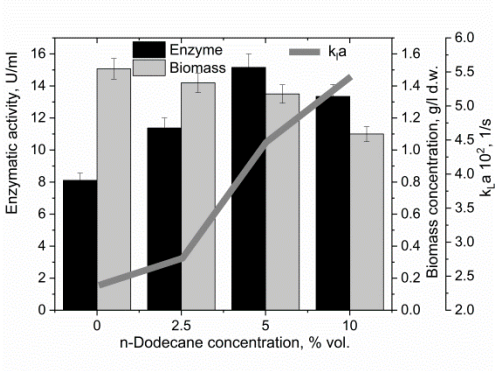
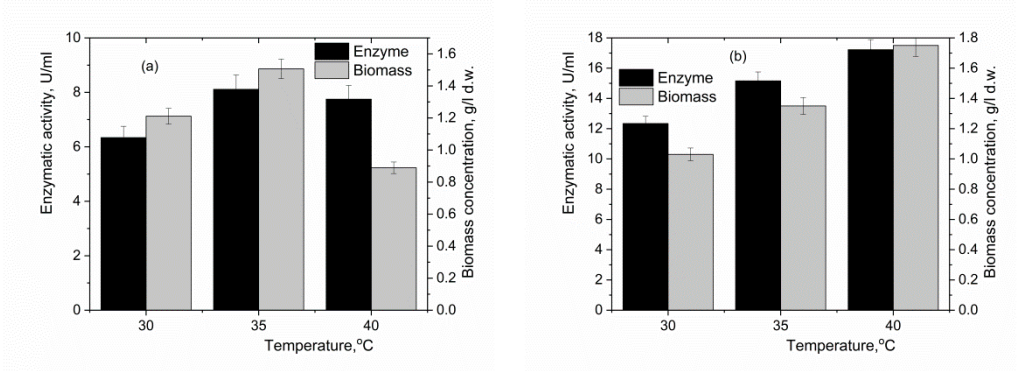


Figure 5. Variation in α-amylase activity and *A. terreus* biomass concentration with *n*-dodecane volumetric fraction on the third day of fermentation (pH = 6).

The effect of the oxygen-vector must also be analyzed in the conditions of fermentation temperature modification. In the absence of the oxygen-vector, the highest amount of biomass was obtained at a temperature of 35 °C, which decreased to half by increasing the temperature to 40 °C (Figure 6a). A similar variation was recorded for α-amylase activity, but the effect of increasing the temperature above 35 °C was less obvious. For this reason, the ratio between enzymatic activity and fungal biomass concentration increased significantly in the range of 30–40 °C, from 5.25 U/kg to 8.70 U/kg.



(**a**) (**b**)

Figure 6. Variation of α-amylase activity and *A. terreus* biomass concentration with temperature on the third day of fermentation without *n*-dodecane (**a**) and with 5% vol. *n*-dodecane (**b**) (pH = 6).

Contrarily, according to Figure 6b, in the presence of 5% vol. hydrocarbon, the increase in temperature showed a positive effect both on the amount of biomass accumulated and on the α-amylase production. The amount of fungal biomass increased with approximately 70% and the enzymatic activity by about 40% in the experimental temperature range. Under these conditions, the ratio between enzymatic activity and fungal biomass concentration was reduced in the range of 30–40 °C, from 12 U/kg to 9.80 U/kg.

Increasing the fermentation temperature determines the dissolved oxygen desorption, with negative effects on both the development of biomass and the production of α-amylase. The addition of *n*-dodecane compensates for the loss of oxygen from the fermentation broth and generates a positive evolution of the fermentation process at temperatures higher than 35 °C. For the suggestive rendering of the cumulative effects of the oxygen-vector and temperature on α-amylase production, the dependence of enzymatic activity on the two factors was plotted in Figure 7, which confirms that the optimal values of the two parameters are: 5% vol. for the hydrocarbon concentration and 40 °C for the fermentation temperature. By comparison with the fermentation system without *n*-dodecane, at 35 °C, the enzyme production increased 2.30 times.

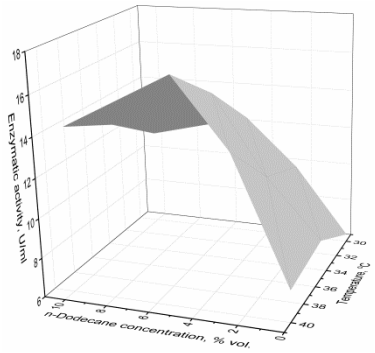


Figure 7. Variation of α-amylase activity with *n*-dodecane volumetric fraction and temperature in the third day of fermentation (pH = 6).

4. Discussion

The *A. terreus* growth under stress conditions, such as very low oxygen concentration or pH, can induce biomass morphological changes and reduce the amount of α-amylase [21]. As previously mentioned, these physiological changes can lead to the production of organic acids, mainly itaconic acid.

The different evolution of the morphology of *A. terreus* pellets in the two cultivation systems, with and without *n*-dodecane, represents the fungi physiological response to the modification of the cultivation conditions [22]. The higher level of dissolved oxygen in the medium, favored by the addition of the oxygen-vector, allows the development of larger spherical pellets, because, in this case, the resistance to internal diffusion of oxygen in the mycelial association is reduced. Under these conditions, the development of fungi continues even in the central regions of the pellets. Basically, due to the development of biomass and higher oxygen consumption, the pellets' growth stops after about four days from the beginning of fermentation, and after five days, the pellets' destruction is observed, due to fungal autolysis in the absence of oxygen and nutrients.

In contrast, in the absence of *n*-dodecane, the concentration of dissolved oxygen and its transfer rate are lower than in the system analyzed above [12,14], which has as the first effect the decrease in the oxygen diffusion rate inside the pellets and, respectively, the appearance of biologically inactive regions in the pellets' central zone, with direct consequences on their development. In response to these conditions, the fungal morphology changes after 2–3 days from the beginning of fermentation: filamentous branches appear, to which the access of oxygen and nutrients is direct, without involving internal diffusion and, therefore, faster. Degradation of the pellets' morphological structure also affects the viscosity of the fermentation broth, because for the same biomass concentration, the filamentous fungal cultures' viscosity is significantly higher than that of pellet fungi [11,23]. Implicitly, the viscosity increase reduces the efficiency of the mixing and transfer processes. As discussed below, morphological structural differences, cumulated with differences in cultivation conditions, generated directly or indirectly, will be reflected in different α-amylase productions.

The addition of *n*-dodecane has an obvious positive effect on α-amylase activity due to the increase in the amount of oxygen available in the environment. In addition, according to Figure 1, the presence of the oxygen-vector attenuates the strong pH reduction, with a beneficial influence on enzyme production. In general, the literature sustains the direct relationship between the amount of fungal biomass and the enzymatic activity [6,8,20]. At the same time, there are situations in which the biomass growth is not directly correlated with the accumulation of the enzyme due to environmental factors (concentration of: sugars, nitrogen sources, or dissolved oxygen, etc.) [1,22]. In this sense, from Figure 5, it can be observed the reduction in the amount of fungal biomass accumulated in the first three days of fermentation with the increase in the *n*-dodecane concentration and, implicitly, with the increase in the dissolved oxygen concentration in the fermentation broth. Basically, for 10% volumetric hydrocarbon fraction, the biomass amount decreased by about 40% compared to the fermentation system without the oxygen-vector.

This variation is the consequence of several phenomena that occur with the increase in the amount of *n*-dodecane in the fermentation broth. Firstly, higher amounts of hydrocarbons can induce fungal growth inhibition, as reported in the literature [26]. Secondly, as it can be observed from Figure 5, the oxygen mass transfer rate, described by oxygen transfer coefficient k_La, is continuously amplified by increasing the *n*-dodecane amount into the broth, a phenomenon that becomes more pronounced for a hydrocarbon volumetric fraction over 2.5%. For 10% vol. oxygen-vector, k_La is about 2.4 times higher than its value in absence of *n*-dodecane. This variation in k_La influences the above discussed aspects related to the biomass morphology or productivity, as it was previously reported for other fungal fermentation processes [12]. However, the acceleration of the oxygen transfer rate from the gaseous phase to the microorganisms, implicitly, the increase in oxygen concentration into the broth can generate, on the one hand, the oxidative stress, and, on the other hand, a more rapid consumption of the substrate, its faster depletion and, implicitly, the limitation in fungal growth [1]. This is also encountered in pressurized systems [27,28].

An interesting and apparent contradictory phenomenon with the literature data is the opposite variation of biomass concentration and α-amylase activity (Figure 5). Thus, if the ratio between enzymatic activity and biomass concentration is considered, it can be noted that it increases from 5.4 U/kg for the fermentation system without *n*-dodecane to 10.8 U/kg for 5% vol. *n*-dodecane, becoming 11.5 U/kg for the system containing 10% vol. hydrocarbon. The results are in agreement with the literature, which mentions the increase in enzymatic activity after fungal biomass development reduction or stopping [1]. Another important aspect, apart from the biomass concentration, is its morphology. The development of fungi in the form of larger pellets, in the presence of the oxygen-vector/higher oxygen concentration, hinders the access of the substrate inside the pellets, which creates conditions similar to the depletion of the substrate in the fermentation broth and promotes enzyme biosynthesis.

5. Conclusions

The biosynthesis of α-amylase by *A. terreus* can be greatly enhanced by the addition of an oxygen-vector into the fermentation broth. Using *n*-dodecane for this purpose, the enzymatic activity after three days from the beginning of fermentation was 1.8–2.0 times higher in the system with hydrocarbon compared to the system without the oxygen-vector, at 35 °C fermentation temperature. Moreover, the presence of the oxygen-vector may partially compensate for the lack of pH control during fermentation and the oxygen desorption from the environment at higher temperatures. At the same time, by adding *n*-dodecane, the fungal morphology can be controlled, the developed pellets having a more compact structure and a larger size.

Compared to the system without *n*-dodecane, in the presence of this hydrocarbon, no direct dependence on the amount of fungal biomass and α-amylase production is noticed, the ratio between enzymatic activity and biomass concentration ranging from 5.4 U/kg for the fermentation system without *n*-dodecane at 11.5 U/kg for 10% vol. hydrocarbon

added in the fermentation broth. The experimental data indicated that the optimal value of the oxygen-vector concentration is 5% vol.; above this level, the oxidative stress or the inhibition generated by a high hydrocarbon concentration is induced. The presence of *n*-dodecane shifted the optimum fermentation temperature to higher values, respectively, 40 °C, mainly as an effect of compensating for the depletion of dissolved oxygen by desorption. Under these conditions, the enzymatic activity was about 2.30 times higher than for the fermentation at 35 °C in the absence of the oxygen-vector.

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