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Assessment of growth rate of *Yarrowiali polytica* and *Pichia guilliermondii* species in an ammonium formate solution of rubber wastewater

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Abstract

The growth of microorganisms is influenced by several physico-chemical parameters that need to be controlled before starting a biological treatment plant for better process efficiency. The influence of temperature (25-40 °C), and ammonium formate concentration (1.59-7.94 mM) on the growth of two yeast strains (*Y.lipolytica and P.guilliermondii*)was examined in a batch process. Temperature has a direct impact on the kinetic growth parameters with an activation energy (Ea) of 14.3 kcal/mol and R^2 0.95 for *Y.lipolytica* and 12.5 kcal/mol and R^2 0.97 for *P.guilliermondii*, indicating a dominant biological regime.

Keywords: Yarrowiali polytica, Pichia guilliermondii, ammonium formate, rubber wastewater, batch bioreactor

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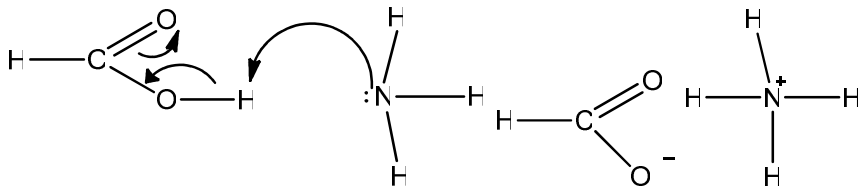
1. Introduction

Ammonium formate ((NH_4HCO_2) is produced in the rubber industry by the reaction of formic acid, used in the factory to coagulate the latex, and ammoniac, which is incorporated into the latex in the field to prevent premature coagulation (Iagba *et al.,* 2008).



Formic acid Ammoniac Formiate Ammonium

(Ammonium formate) HCO_2NH_4

Ammonium formate is an ammonium salt with exposure effects that vary with concentration. Transient and reversible health effects are observed at 3.7 g/l. From 41 g/L, the effects become adverse and irreversible (severe irritation and permanent damage to the eyes, irritation of the mucous membranes and respiratory tract, stomach pain and difficulty breathing and finally gastrointestinal irritation) and death occurs above 240 g/L, the lethal dose (SCAPA, 2016; USEPA, 2016). Furthermore, when this compound dissolves in water, it produces two species (ammonium ions and formate ions) that are environmentally toxic either directly or through their metabolites. Water containing the ammonium ion causes the formation of carcinogenic nitrosamines, the formation of methemoglobin in infants, and the inhibition of certain digestive and respiratory tract pathways (Nsoe *et al.,* 2016; Sulaiman *et al.,* 2010; Arimoro, 2009; Bourgard, 2004). Environmental eutrophication of mangroves causes dissolved oxygen depletion and consequent death of aquatic biological life (Dibyendu and Homagn., 2016; Tekasakul, 2010).

Increased ammonium ion concentrations in the environment cause long-term imbalance in the nitrogen cycle and accelerated denitrification, which can increase concentrations of nitric oxide and nitrous oxide, both of which contribute to ozone depletion (Viard*et al.,* 2013). The reduction of formations produces CO_2, which is responsible for more than 60% of the greenhouse effect (Ndi*et al.,* 2016). Given all of the health and environmental concerns associated with this compound, it is critical to treat the effluent from rubber latex coagulation before it is released into the environment. Lagoons and oxidation pits are biological processes to treat rubber industry effluents without considering abiotic and biotic factors (Ndi*etal.,* 2016, Mitra*etal.,* 2010; Tekasakul., 2010). The contact time between the microorganism and the pollutant load therefore requires a long residence time, release of odors and non-compliance with legal discharge limits. Therefore, it is important to consider these factors. Abiotic and biotic factors influence biological wastewater treatment by promoting or inhibiting the development of microorganisms responsible for the biodegradation of pollutants (Dehghani and Hassan., 2013). Controlling and understanding these factors is beneficial both in the design of wastewater treatment plants and in improving and understanding microbial activity to ensure purification performance (Milton, 2009; Songming and Shulin 2002; Bo and Swantje, 1998; Harvey and George 1991).

The main factors affecting treatment efficiency are pollutant concentration, temperature and pH. (Fqih *et al.,* 2000; Nedwell, 1999). The main factors affecting treatment efficiency are pollutant concentration and temperature. (Fqih *et al.,* 2000; Nedwell, 1999). Every microorganism has a pollutant tolerance threshold. Above this point, the pollutant becomes an inhibitor, slowing the growth of microorganisms. For this reason, understanding the concentration range is crucial in order to optimize treatment (Dutta *et al.,* 2014). Temperature can act as a catalyst or inhibitor depending on the environment. Yeasts develop resistance and become spores at low temperatures, which reduces their metabolism and thus the cleaning performance of the stations. On the other hand, at high temperature, there is reversible dissociation and irreversible denaturation of proteins at the membrane level, which can lead to yeast death and low purification yield (Milton, 2009; Fqih *et al.,* 2000). From now on we will study the effect of Ammonium formate, concentration and temperatureto determine the optimal conditions for the growth of yeast species (*Yarrobialipolitica* and *Pichia guillermondii* separately from Nsoe *et al.,* (2013) to determine. from nature, rubber plant waste and has demonstrated the ability to grow in rubber ducts.

2. Materials and methods

2.1. Yeast inoculum

The yeast strains used in this work were isolated from rubber industry waste thanks to the Nsoe protocol (Nsoe*et al.,*2013). Microbial properties are listed in Table 1.

Table 1: Characteristics of yeast species(Nsoe*et al.*, 2018)

Code	Yeast colour	Zeta Potential pH (5,7-7)	Yeast shape	Yeast size (µm)	Growth in selective media	
					Acid medium	Alkaline medium
Yarrowialipolytica	Red	-25,3 to -37,8	Ovoid	0,3-150	+	+
Pichia guilliermondi	White	-27,8 to-30,5	Ovoid	0,2-100	+	+

Yeast was cultivated on the following growth medium (Atagana, 1996): meat extract 1 g. L^{-1} (Sherlanrf 07-075, Spain), yeast extract 2 g. L^{-1} (OXOID code L21, England), peptone 5 g. L^{-1} (Liofilchem rf 610038, Italy), sodium chloride 5 g.L^{-1} (Jeulinrf 107115, France) at pH = 6.5.

2.2. Synthetic influent

A synthetic influent was created to ensure the concentration common in the rubber industry. Ammonium formate is the sole source of carbon and nitrogen. This was added to cover the varying nitrogen content: from 1.59 to 7.94 mM (22.3 to 111 mgN.L-1).

Ammonium formate was mixed with mineral salt medium (in $g.L^{-1}$): $MgSO_4$ (0.2), CaCl (0.02), KH_2PO_4 (60), K_2HPO_4 (14). A stock solution of microelements (in $g.L^{-1}$): $ZnSO_4$(10.90), $FeSO_4$(5), $MnSO_4$(1.54), $CuSO_4$(0.39) was prepared and added to the influent at 0.1% (v/v). Then 0.25 g of L^{-1} chloramphenicol was added to inhibit bacterial growth and the synthetic influent was sterilized at 110°C for 10 minutes.

2.3. Experimental set–up: Batch bioreactor

Figure 1 shows us the batch bioreactor assembled for this experiment, it consists of several 1L bioreactors (with a usable volume of 0.8L) that were used and immersed in a water bath to keep the temperature at 25C. Aeration was supplied thanks to an air compressor (GIANESSIEDILIO, NML 58629, LT 100, ATE 12TEMPA, Italy) to ensure that dissolved O_2 was not a limiting factor for respiration and biomass growth. To reduce bacterial contamination and CO_2 levels, the air inlet is subjected to a series of washes containing 1mM sodium hydroxide and 1mM hydrochloric acid. Then, the air outlet was connected to a 500 mL vial containing a 0.5 mM sodium hydroxide solution to quantify the CO_2 released during the degradation of ammonium formate.

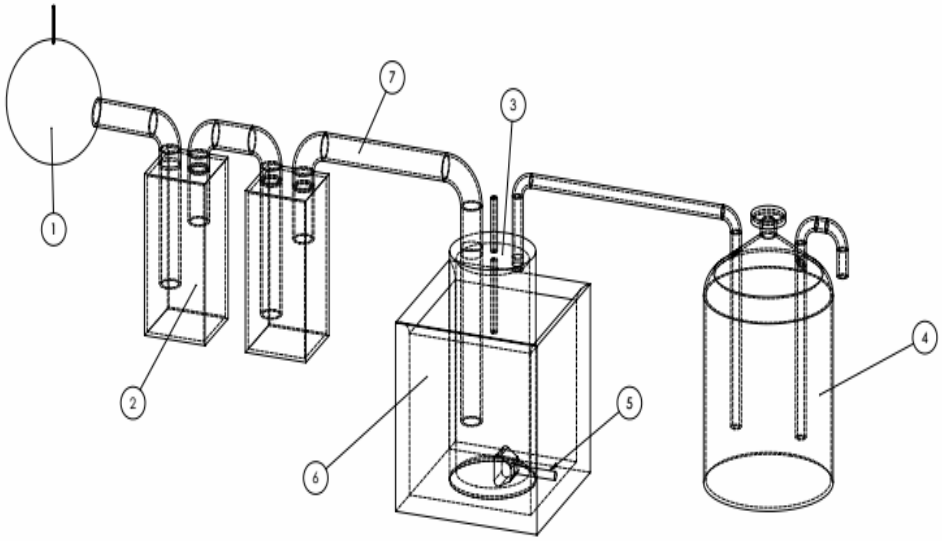


Figure 1: Batch bioreactor (1. Compressor; 2. Air cleaner); 3.Bioreactor;4. CO_2 removal; 5.Thermoregulator; 6. Water bath

2.4. Influence of ammonium formate concentration on growth

Fig1 shows us the batch bioreactor assembled for this experiment, it consists of several bioreactors of 1 L (with a useful volume of 0.8 L) used and immersed in a water bath to keep the temperature at 25 °C. Aeration was supplied thanks to an air compressor (GIANESSIEDILIO, NML 58629, LT 100, ATE 12TEMPA, Italy) to ensure that dissolved O_2 was not a limiting factor for respiration and biomass growth. To reduce bacterial contamination and CO_2 levels, the air inlet is subjected to a series of washes containing 1mM sodium hydroxide and 1mM hydrochloric acid. Then, the air outlet was connected to a 500 mL vial containing a 0.5 mM sodium hydroxide solution to quantify the CO_2 released during the degradation of ammonium formate.

2.5. Activation energy

The effect of temperature on microbial growth was evaluated via the activation energy obtained using the Arrhenius equation (Kirchman, 2012; Attilio and Jose, 2001). This relationship describes the general dependence of the rate of a reaction on thetemperature and is given by equation 1.

$$\mu_{\max} = \mu_0 e^{\left(\frac{-E_a}{RT}\right)} \quad (1)$$

With:

E_a: Activation energy of microbial growth, (kcal/mol); $\mu_{0:}$ pre-exponential factor (h^{-1}) ; R : gas constant (kcal/mol. K); T: Temperature (K).

2.6 Q_{10} *coefficient*

This parameter represents the increase in reaction rate when there is 10°C increase in temperature. The value of Q_{10} is calculated using equation 2 (Urbano et al., 2005):

$$Q_{10} = \left(\frac{K_2}{K_1}\right)^{\left(\frac{10}{T_2 - T_1}\right)} \quad (2)$$

T_2 :Upper temperature (°C); T_1 :Lower temperature (°C); μ_2 :Higher reaction speed μ_1 :Lower reaction speed

3. Results and discussion

3.1. Yeast kinetic growth

Figures 2 and 3 individually show the evolution of the population of strains of *Yarrowiali polytica* and *Pichia guillermondii* as a work of time. These engine bends all have classic yeast development profiles for both species (initial stage, accelerated stage, decaying stage, stationary stage).

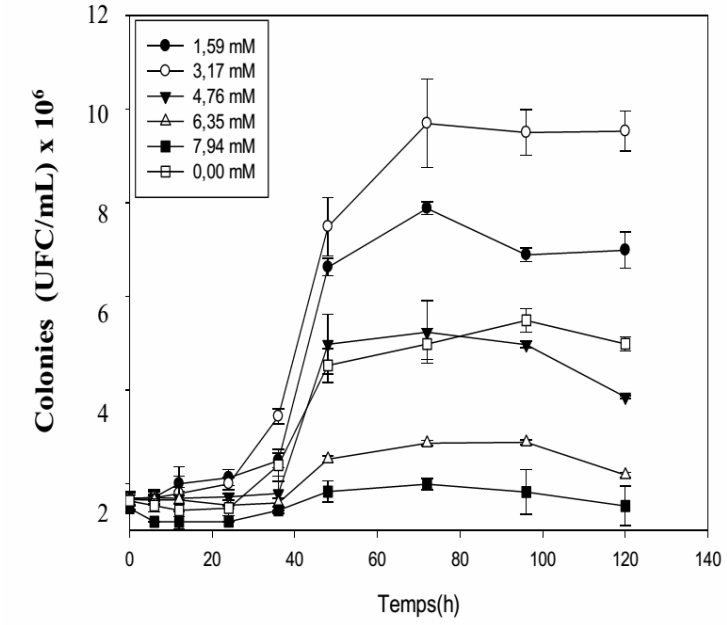


Figure 2 : Influence of the concentration of ammonium formate on the growth of specie *Yarrowialipolytica* as a function of time at pH 6 and 28 ± 2°C

Yeast growth varies with the concentration of ammonium formate independent of the strain. In the two species, two phenomena were observed when the formate concentration was greater than 3.17 mM; as the latency period increases, the growth of the yeast slows down. According to Amrouche*et al*. (2011), the increase in the latency period is an adaptation of the yeast to high concentrations of the substrate. This increase in latency can also be caused by the obstruction of the enzyme's catalytic center by the excess substrate, or alternatively, the substrate can lodge in the active site with an abnormal orientation, preventing the reaction from proceeding; This is the reason for the low yield of yeast production caused by low number of metabolized substrates due to inhibition. On the other hand, when the formate concentration is less than or equal to 3.17 mM, vigorous growth of microorganisms was observed due to availability of the substrate. However, at concentrations less than 3.17 mM, this growth is weaker than that obtained from the concentration of ammonium formate at 3.17 mM. This is because the 3.17mM maximum concentration is the concentration for optimal yeast growth.

Thus, the ammonium formate concentration at 1.59 mM is insufficient to ensure maximum yeast growth due to substrate depletion. In order to better visualize the influence of the concentration of ammonium formate on the growth of yeast, a representation of the influence of the concentration on the maximum growth rate is created.

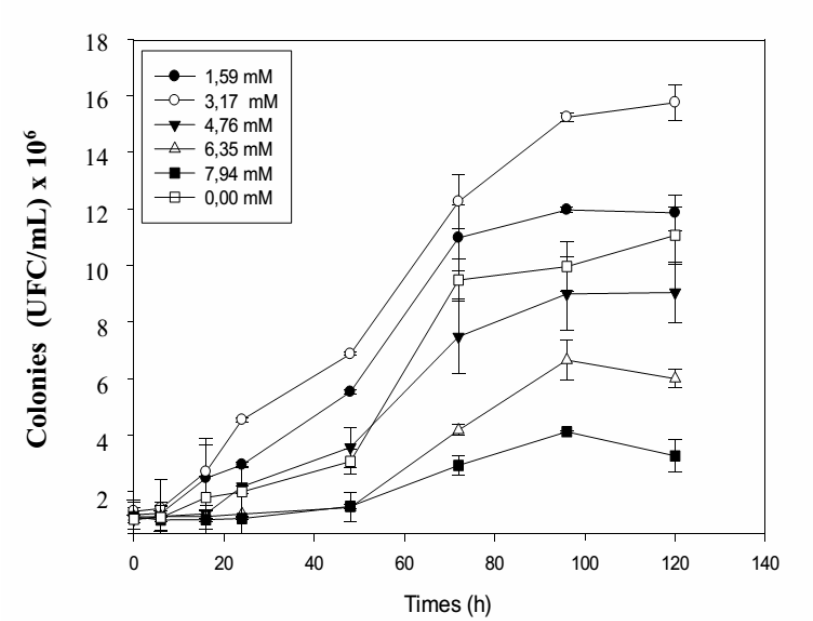


Figure 3: Influence of the concentration of ammonium formate on the growth of the *Pichia guillermondii*specie as a function of time at pH 6 and at 28 ± 2°C

3.1.1. Maximum growth rate of yeasts at different concentrations of ammonium formate

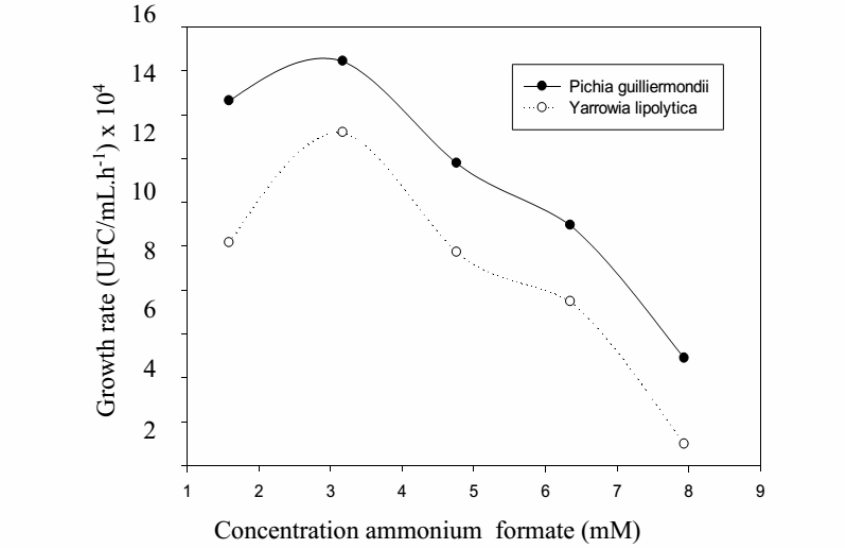


Figure 4: Influence of initial ammonium formate concentrations on the maximum growth rate of *Yarrowialipolytica* and *Pichia guilliermondii* species, at pH 6, 28 ± 2°C

The *Pichia guilliermondii* specie shows a maximum development rate (UFC/mL/h-1) of 18*104. This speed is 1.21 more remarkable than the maximum of specie*Yarrowialipolytica* which is 15*104. This may be due to a strong adaptation of the *Pichia guilliermondii* specie to ammonium formate, which shortens the adaptation time, or it may be due to a less complex digestive system compared to the *Yarrowialipolytica* specie. To confirm this, we determine the duplication factor.

3.1.2. Yeast growth multiplication factor as a function of ammonium formate concentration

Although the maximum growth rate of *Pichia guilliermondii* is higher than that of *Yarrowialipolytica, Yarrowialipolytica* has been found to have a 60% higher biomass production rate than *Pichia guillermondii*. This shows us that *Y. lipolityca* reproduces rapidly compared to *P. guillermondii.* The optimum growth rate and multiplication factor for both strains is 3.17 mM, which is

higher than the maximum concentration found in rubber mill effluents. In order to investigate the influence of other parameters, this concentration is recorded and kept constant.

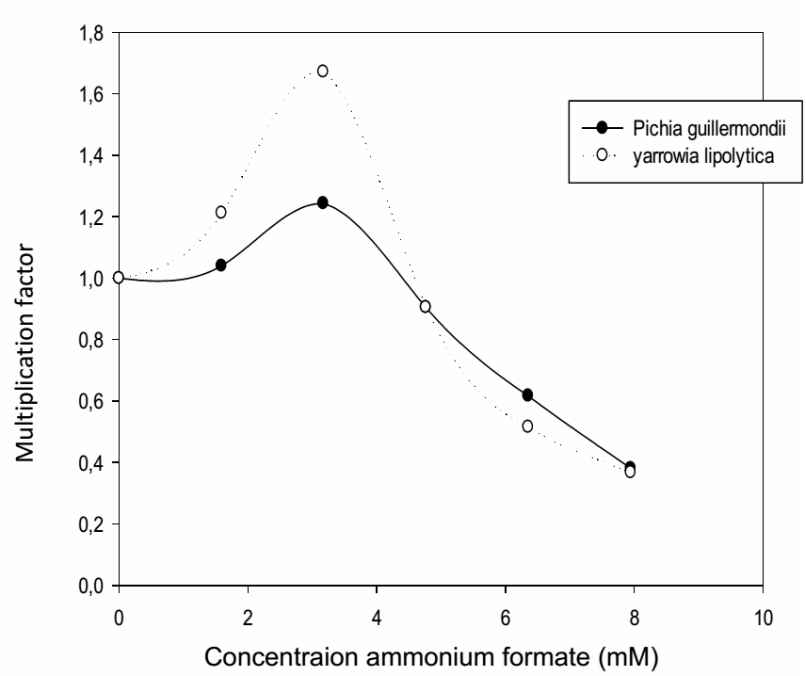


Figure 5: Variation of the multiplication factor as a function of the concentration of ammonium formate in yeast species (*Pichia guilliermondii* and *Yarrowialipolytica*) at 96 h, pH 6, 28 ± 2°C

3.2. Impact of temperature on yeast growth

Temperature is one of the important factors to consider when studying yeast growth. It can actually modify growth kinetic profiles; therefore we have highlighted its influence on specific growth rate, activation energy and Q_{10}.

3.2.1. Yeast growth kinetics as a function of temperature

For the two yeast species studied, we followed the growth of the yeasts throughout the biodegradation of ammoniumformate. As shown in Figures 6 and 7 for the two yeast strains.

Figs 6 and 7 show the obtained biomass production profiles as a function of time for temperatures of 25, 30, 35 and 40°C. Although the growth of the biomass presents the same phase (starting phase, acceleration phase, deceleration phase, stationary phase), regardless of the temperature and the stress between 25 and 30 ° C, the temperature has a positive influence on the yeast growth due to a latency time that is independent of the yeast strain is almost zero. In this temperature range, the production of biomass is maximum, but varies from strain to strain. This observation confirms that the yeasts were activated upon inoculation and that the preculture conditions were satisfactory. The heat from the environment provides additional energy that facilitates enzymatic reactions, leading to an increase in biomass and consequently a reduction in the residence time of the pollutant in the bioreactor. On the other hand, between 35ºC and 40ºC we observe a slowdown in the production of biomass, with a latency that increases with temperature and differs from strain to strain. This rather long latency period may reflect an increase in biodegradation time and poor biodegradation of the organic matter. These results can be compared to those obtained by various authors on the effect of temperature on yeast growth (Torija*et al.,* 2002). Lucero *et al.,* (2000) believe that increasing the temperature leads to damage to the structure of the cell membrane and consequently to a reduction in its transmission properties. Denaturation of the secondary and tertiary structures of the enzyme can also occur.

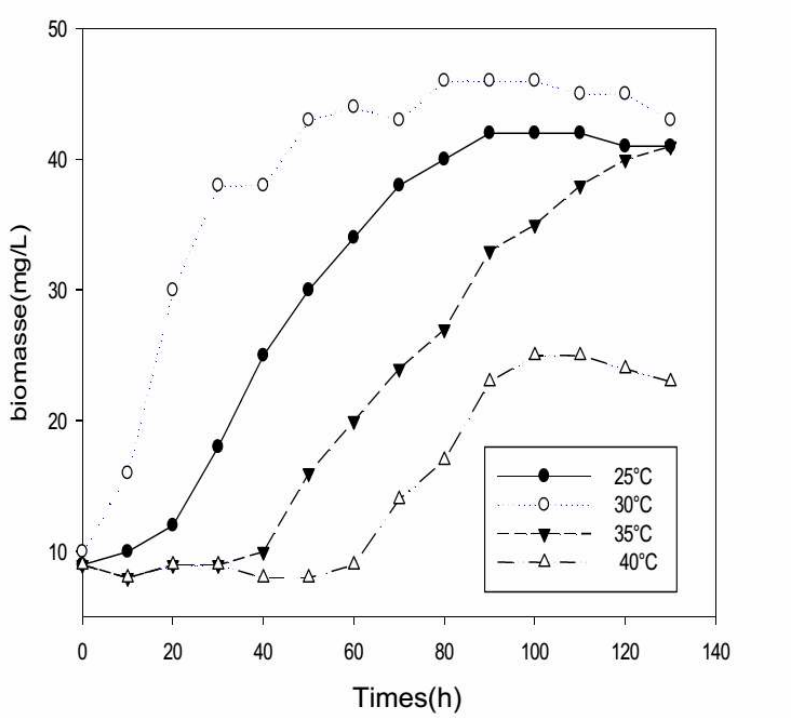


Figure 6 : Variation of biomass as a function of time at different temperatures and at a constant concentration (3.17 mM) of ammonium formate for the specie*Yarrowialipolytica*, pH 6

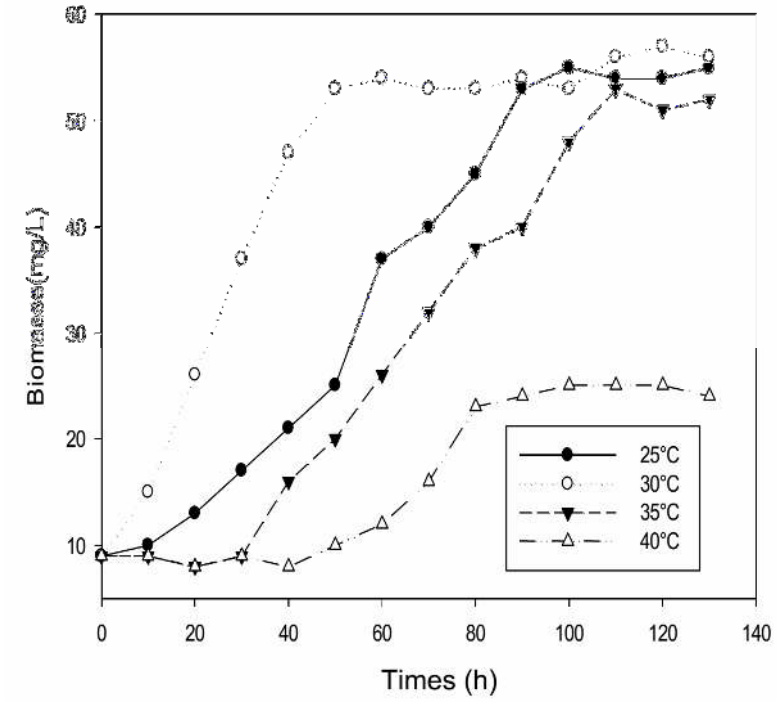


Figure 7: Variation of biomass as a function of time at different temperatures and at a constant concentration (3.17 mM) of ammonium formate for the specie*Yarrowialipolytica*, pH 6

3.2.2. Influence of temperature on specific growth rate

It can be seen that the specific growth rates are strongly influenced by temperature. Regardless of the strain, the specific growth rate decreases with increasing temperature. With maximum values at 28°C in the *Pichia guilliermondii* strain (0.112 h^{-1}) and at 25°C in the *Yarrowialipolytica* strain (0.102 h-1). These results agree with the work of Torija*et al.*(2002), who confirm the

negative effects of temperature on yeasts. For a better cleaning performance and for the cultivation of the yeast, it is advisable to work in the range of 25 to 30 °C.

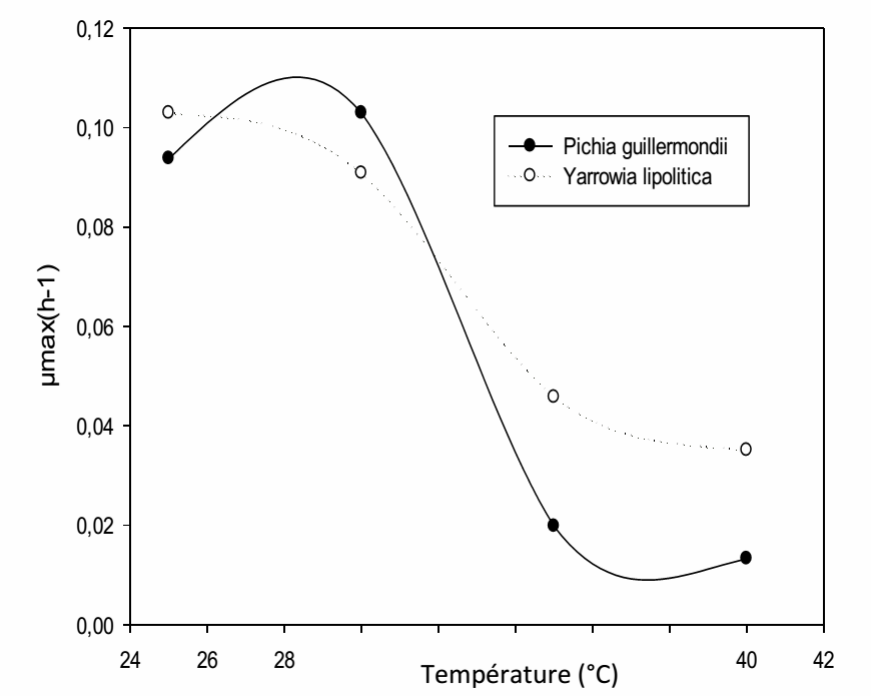


Figure 8: Variation of the specific growth rate (µmax) as a function of temperature for *Yarrowialipolytica* and *Pichia guilliermondii* species

3.2.3. Activation energy

The Arrhenius equation describes the general dependence of the rate of a reaction on temperature (Cisse *et al.,* 2009) as shown in Figure 9.

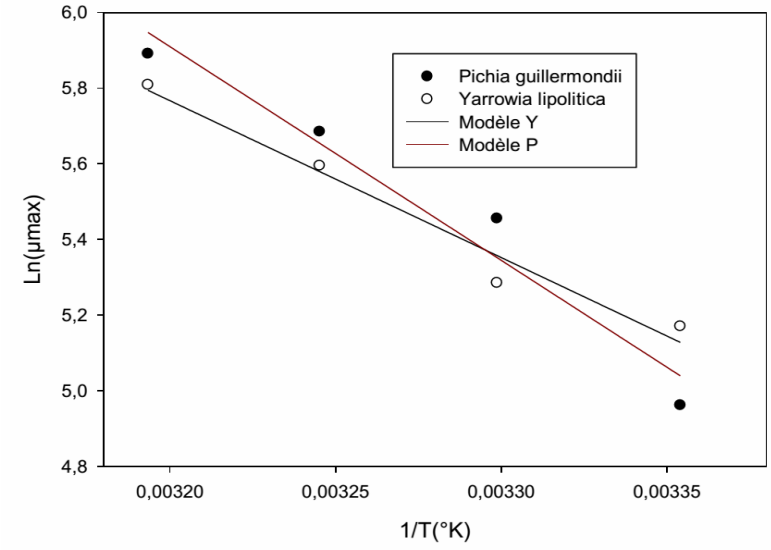


Figure 9: Logarithm of the maximum specific speed of growth at different temperatures (Application of the Arrhenius equation) for the species*Yarrowialipolytica* and *Pichia guilliermondii*

Table 2: Activation energy (Ea), and regression coefficient (r^2) for the two yeast species.

Microorganismes	Ea (kcal/mol)	r^2
Yarrowiali polytica	11,3	0,95
Pichiaguilliermondii	8,3	0,97

We determined the parameters of the Arrhenius equation for the two strains using the equations on the right side of Figure 9, which represents the natural logarithm of the maximum growth rate (biomass production) at different temperatures. The values of the activation energy (Ea) and the correlation coefficient r2 are recorded in Table 2. The activation energies obtained are 11.3 and 8.3 kcal/mol, respectively, for the species*YarrowiaLipicica*and *Pichia guilliermondii* with r^2 of 95 and 97. From the values it is concluded that *Pichia guilliermondii* is less sensitive to temperature than *Yarrowialipolytica*. The latter requires more energy to carry out its metabolic reactions. With an activation energy value equal to or greater than 12 kcal/mol, the activation process is in a biological regime (Sanchez *et al.,* 2004; Serra *et al.,* 2005). We conclude that we are in a biological regime for *Yarrowialipolytica* and in a diffusive regime for the specie*Pichia guilliermondii.*

3.2.4. Q10 *factor*

In the same way as for the activation energy, the value of Q10 can be used to know if the process is physical (Q10≤ 1) or biochemical (Q10 ≥2) (by diffusion or biological). The Q10 coefficient is also a useful tool to indicate the sensitivity of the response to an increase in temperature within a defined range by measuring changes in growth rate, as shown in Table 4.

Table 3: Valeur du Q_{10} pour *Yarrowialipolytica* et *Pichiaguilliermondii*

Yeast	Q_{10} (entre 25-35°C)	Q_{10} (entre 30-40°C)
Yarrowiali polytica	5,07	3,32
Pichia guillermondii	1,88	1,19

Table 3 shows that the Q10 factor is a function of strain and temperature. Between 25 and 35 °C the Q10 value is 1.88 for *Pichia guilliermondii* and 5.07 for *Yarrowiali polytica*. While between 30 and 40 C this value decreases for the two species*Pichia guilliermondii*(Q10= 1.19) and 3.32 for *Yarrowiali polytica*. According to Apple *et al.,* (2006), Sand-Jensen *et al.* (2007), Q10 values are higher at low temperatures because under such conditions the biochemical reactions involved are limited by a decrease in enzymatic activity. On the other hand, at high temperatures (above the threshold temperature) the value of Q10 decreases and under these conditions a physical limitation occurs, for example the decrease in oxygen diffusion.

4. Conclusion

The ability of *Yarrowiali politica* and *Pichia guillermondii* to grow in a medium rich in ammonium formate was studied. It shows that the concentration of ammonium formate is observed at a maximum growth of *P.guilliermondii* and *Y.lipolytica* of 3.17 mM, which is higher than the concentrations of ammonium formate found in the rubber effluent. Temperature has a direct impact on yeast growth with a maximum between 25 and 30°C. The formate molecule can be either a substrate or an inhibitor depending on the concentration. In order to know the molecule responsible for the inhibition and the types of inhibition, it would be advisable to study the biodegradation kinetics of the formate and the formate present in the medium.

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