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Review

Plant latex lipase as biocatalysts for biodiesel production

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Industrial-scale processes currently developed make use of chemical catalysis processes that are highly efficient but require very complex product purification steps. Enzymatic catalysis through plant lipases as biocatalysts is an alternative which, in contrast to chemical catalysis processes, appeared simple to perform, and can be done at low investment cost. Although microbial lipases have been extensively studied, little research has been focused on the use of plant lipases namely plant latex lipases. The present article outlines the most advanced knowledge concerning plant latex characterization in order to show how plant latex can be a promising alternative to catalyze transesterification for biodiesel production. This paper provides an overview regarding the main aspects of latex, such as the reactions catalyzed, physiological functions, specificities, sources and their industrial applications.

Key words: Plant latex, lipase, Transesterification, purification, biodiesel.

INTRODUCTION

Interest in the production of biodiesel a clean renewable fuel is increasing worldwide due to the excessive increase of petroleum prices and the importance of taking environmental concerns into consideration (Mounguengui et al., 2013; Sadeghinezhad et al., 2013). Bio-fuel or biodiesel is usually identified as ester based fuels produced from animal fats or from vegetable oils by using an effective transesterification method. Biodiesel carries 4.5 units of energy against each unit of fossil fuel (Pradhan et al., 2009). Besides this, biodiesel is safer, biodegradable and nontoxic in nature (Mc Carthy et al., 2011). The mixtures obtained after transesterification are composed of fatty acid alkyl monoesters (Robles-Medina et al., 2009; Meher et al., 2006) and to be classified as "biodiesel", it must achieve minimum purity and fulfil the specifications of international standards (Graboski et al., 1998), the European standard EN14214 (FAME, 2003) and the American standard ASTM 6751-09 (ASTM,

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2009). Fatty acid alkyl monoesters may be methyl or ethyl esters respectively if the alcohol used is methanol or ethanol. Conventional biodiesel production methods involve the use of acid or base chemical catalysts *via* homogenous or heterogeneous processes (Marchetti et al., 2007; Brunschwig et al., 2011). Downstream processing costs and environmental problems associated with biodiesel production and byproducts recovery have investigated many research teams to develop new alternative catalysis processes to replace the homogenous catalysis processes generally used in industries (Akoh et al., 2007). In contrast, enzymes (lipases) allow the production of specific alkyl esters, easy recovery of the glycerol, and transesterification of TAG with high free fatty acid content (Soumanou et al., 2012).

Lipases have become more and more prominent on the enzyme biotechnology scenario due to their versatility for hydrolysis and synthesis, their catalytic reactions often being chemo-selective, regio-selective or enantio-selective. Lipases are used in many sectors such as the food, pharmaceutical, fine chemical, oil chemical, biodiesel and industrial detergent industries (Alonso et al., 2005). Lipases act, by definition, at the organic-aqueous interface, catalyzing the hydrolysis of estercarboxylate bonds and releasing fatty acids and organic alcohols (Pereira et al., 2003; Leal et al., 2002; Kamimura et al., 1999; Merçon et al., 1997). However, as Pottevin showed for the first time in 1906, in water-restricted environments, the reverse reaction (esterification) or even various transesterification reactions can occur (Castro et al., 2000).

Lipases can be of animal (pancreatic, hepatic and gastric), microbial (bacterial, fungal and yeast) or vegetable origin, with variations in their catalytic properties (Mukherjee and Hills, 1994). To date, microbial lipases are the most studied. In fact around 58% of the publications of the whole of lipases are devoted to microbial lipases, plant lipases are around 42% of publications and only 11% of the publications are devoted to latex lipases (Google Scholar, 2014). Nevertheless, despite the extensive range of microbial lipases, the use of these enzymes on an industrial scale is still restricted due to high production costs, favoring the search for other sources of these enzymes (Parques and Macedo, 2006).

The modification of fats and oils by transesterification, for instance, can be performed by both chemical and enzymatic catalysis. The industrial transesterification process is currently performed by chemical means, using high temperatures and alkaline metals (KOH, NaOH, HCl, H_3PO_4, H_2SO_4… etc) as the reaction catalyst (Ribeiro et al., 2009; de Araújo et al., 2013). In the enzymatic process, lipases can be used as biocatalysts to promote the exchange of triacylglycerols, showing greater efficiency and leaving no residues (Xu, 2000). For example immobilized *Candida antarctica* lipase has been used for ethyl esterification of docosahexanoic acid and later used to effect over 98.5% fatty acid methyl ester conversion (Fjerbaek et al., 2009). Latex lipases present certain advantages since they do not necessarily have to be purified in order to perform this and other processes (Cambon, 2008). However, several studies have indicated that such processes are very expensive due to the high cost of purification step (de Castro et al., 2004; Noor et al., 2003). Recently, latex lipases have been the focus of much attention as biocatalysts. In some cases, these enzymes present advantages over animal and microbial lipases due to some quite interesting features such as specificity, low cost, availability and ease of purification, representing a great alternative for potential commercial exploitation as industrial enzymes (Villeneuve, 2003).

Although microbial lipases have been extensively studied (Kilcawley et al., 2002; Mendes et al., 2012), little research has been focused on the use of plant lipases in biodiesel production. However, the major drawback for plant lipases implementation at large scale is the low content of enzyme in the post-germination seeds. However, *Caricaceae* or *Euphorbiceae* overcome this disadvantage as their enzymes are available in large amounts stored in their latex (Paques and Macedo, 2006).

The aim of this review was to highlight the potential and current limitations to the use of plant latex lipases for low-cost enzymatic catalysis alkyls esters production and therefore for a possible application for biodiesel production.

LATEX SOURCES AND PLANT-BASED LATEX

Latex is an aqueous emulsion or a milky fluid (Mounguengui et al., 2013) found in the vacuole of specialized secretory cells known as ‘‘laticifers’’ (Fahn, 1982). Some family plants known as laticifers are *Apocynaceae, Asclepiadaceae, Euphorbiaceae, Moraceae* and *Sapotaceae* (Moulin et al., 1994; Palocci et al., 2003; Giordani et al., 1991; Villeneuve et al., 2005; Dhuique-Mayer et al., 2001, 2003). The laticifers plants show intense metabolic activity, with the latex containing lipids, rubbers, resins, and sugars, as well as several proteins and many different enzymes (that is, peroxidases, proteases, esterases, and phosphatases) (Fiorillo et al., 2007; El Moussaoui et al., 2001; Lynn and Clevette-Radford, 1987). The laticifers plants also show secondary metabolic activity directed toward the production of defence-related molecules, which accumulate in appreciable amounts in the latex. Many toxic substances known to be stored in the latex (that is, alkaloids, sterols and terpenoids) have been shown to have a negative impact on insect feeding and phytophage fitness (El Moussaoui et al., 2001). The latex thus represents a chemical defence, and the physiological

role of its constituents, including lipolytic enzymes, could be related to defence mechanisms (El Moussaoui et al., 2001).

The water insoluble fraction of latex shows lipase activity. It is now known that lipases contained in latex from some plants have catalytic properties and numerous industrial applications (Paques and Macedo, 2006). For instance, papaya (*Carica papaya*) latex has already been described in the modification of fats and oils (Villeneuve, 2003; Foglia and Villeneuve, 1997a), in esterification and transesterification reactions (Caro et al., 2000), and more recently in the resolution of racemic mixtures (Cheng and Tsai, 2004). Studies carried out on *C. papaya* showed that its crude latex has a very strong activity on short-chain triacylglycerol (TAG) and *sn3* stereoselectivity in hydrolysis reactions of chiral TAG substrates (Villeneuve et al., 1995). These observations led to industrial use of this latex in particular applications, such as the synthesis of low-calorie triacylglycerols (Foglia and Villeneuve, 1997a) or medium-chain TAG (Caro et al., 2004). These results have prompted interest in other latex plant extracts, in particular, the unripe fruit of the babaco plant (*Vasconcellea* X *Heilbornii* cv., ex *Carica pentagona Heilbornii*) (Kyndt et al., 2005), a member of the papaya family native to the subtropical mountains of Ecuador, which contains a latex similar to the one in *C. papaya*. In common with the latter, babaco has also been shown to exhibit biocatalytic activities in lipolysis and acyl transfer reactions (Dhuique-Mayer et al., 2001, 2003). Latex lipases from plants in the *Euphorbiaceae* (Moulin et al., 1994; Palocci et al., 2003; Giordani et al., 1991; Villeneuve et al., 2005), *Asclepiadaceae* (Giordani et al., 1991) or *Caricaceae* (Giordani et al., 1991; Dhuique-Mayer et al., 2001, 2003) families have also been described as useful biocatalysts for several synthesis applications in the food, pharmaceutical and detergent industries.

Chemical composition and molecular structure of plant-based latex

Most plants from families like *Euphorbiaceae* (Giordani et al., 1991; Moulin et al., 1994; Palocci et al., 2003; Villeneuve et al., 2005), *Asclepiadaceae* (Giordani et al., 1991), *Brassicaceae* (Hills et al., 1990), or *Caricaceae* (Giordani et al., 1991; Dhuique-Mayer et al., 2001, 2003) contain latex and have been described as useful biocatalysts for several applications. Here, a summary of chemical composition of some plant-based latex was performed.

Caricaceae

Within the *Caricaceae* family, *C. papaya* is a soft-stemmed and unbranched tree able to grow up to 20 m in height. As a native to the Central America, the *papaya* tree has successfully established in many ecological niches in tropical and subtropical climates (El Moussaoui et al., 2001). All aerial parts of the female and hermaphrodite plants, including unripe fruits, present laticifers (El Moussaoui et al., 2001) that create a dense network of articulated and anastomosing structures (Roth and Clausnitzer, 1972). Consequently, if incisions are made in those aerial parts, especially in the unripe fruits, an abrupt release of latex (the so-called *C. papaya* latex) is observed. This latex is a thixotropic fluid with a milky appearance, which contains around 15% of dry matter, 85% water, and a great variety of hydrolytic enzymes, mainly proteases (El Moussaoui et al., 2001; Azarkan et al., 2003; Campillo-Alvarado and Tovar-Miranda, 2013). Furthermore, different protein compositions have been reported depending on the age or sex of the tree, as well as on the time of day in which the tapping is carried out (Luis Madrigal et al., 1980; Caro et al., 2000). The mixture of different enzymes present in the latex is supposed to play a defensive role in the plant (El Moussaoui et al., 2001).

Babaco (*C. pentagona Heilb*.) is a tropical plant from a mountain climate, native to Ecuador, appreciated for its flavor. Babaco is a perennial shrub of the Caricaceae family. This natural hybrid grows between 1500 and 2500 m in Ecuador. Annual yields are between 60 and 80 fruits per plant. It is a large seedless fruit, yellow when ripe. Its special aroma is described as having overtones of pineapple, lemon and papaya. When the fruit is green, it exudes latex which has proteolytic characteristics similar to the papaya latex. The fruit is directly consumed when it is fully ripe. It is also possible to produce juice concentrate, jam or dehydrated fruit. The extraction of proteolytic enzymes of the latex from green babaco could be a new industrial application for this fruit. Babaco has economical potential thanks to its sensorial properties, and as a source of proteolytic enzymes.

Apocynaceae

In addition to *Apocynaceae* family, *Plumeria rubra* is also studied. It's growing in tropical and sub-tropical regions of the world (Ye et al., 2009; Coppen and Cobb, 1983) and is grown for ornamental purposes (Perry and Metzger, 1980). Plants of genus *Plumeria*, had their origin from Central America. Different species are now found widely distributed in the warmer regions of the world (Krishnamurthi, 1969) and reputed for their medicinal properties, e.g., antifouling (Coppen et al., 1983), anticancer (Fujimoto and Made, 1988), algicidal (Coppen, 1983). The aqueous extract of *P. rubra* showed antimicrobial (Gupta et al., 2007) anti-inflammatory activities (Dubois and Rezzonico, 2007) and used for the treatment of respiratory ailments (Frei et al., 1998; Case et al., 2006). Plumericin, an iridoid isolated from *P. rubra*

is used as antimicrobial agent (Little and Johnstone, 1951).

The average composition of carbon, hydrogen and nitrogen (%) of *P. rubra* was respectively 44.89, 6.72 and 1.26%. Indeed, some studies have reported that a_w conditions the lipase structure and thus its enzymatic activity both in hydrolysis and in synthesis reactions (Cambon et al., 2006).

Moraceae

Ficus carica, (*Moraceae* family), is one of the earliest cultivated fruit trees and an important crop worldwide for both dry and fresh consumption. It is a native of the Mediterranean coast. In these countries, figs are an important constituent of the Mediterranean diet, which is considered to be one of the healthiest and is associated with longevity (Trichopoulou et al., 2006). Oliveira et al. (2009) compared *F. carica* leaves, pulps and peels. Data obtained indicate that chemical composition and bioactivity are dependent on the variety (Oliveira et al., 2009). 3-O-Caffeoylquinic acid and quercetin 3-O-glucoside were described for the first time, which adds to the knowledge of this species. Leaves may constitute an excellent dietary and economical source of bioactive compounds, namely, phenolic compounds.

F. carica latex is essentially constituted by saturated fatty acids (ca. 86.4% of total fatty acids), whilst dried and fresh fruits show predominantly polyunsaturated fatty acids (ca. 84 and 69% of total fatty acids, respectively) (Jeong and Lachance, 2001; Pande and Akoh, 2010). With respect to monounsaturated fatty acids, oleic acid is presented as the most abundant one in latex (Oliveira et al., 2010), which is in agreement with data found for *F. carica* fruit (Jeong and Lachance, 2001; Pande and Akoh, 2010). Concerning polyunsaturated fatty acids, linoleic acid was the only compound identified (ca. 9.9% of total fatty acids) (Oliveira et al., 2010), which was already described in dried and fresh fig fruits (Jeong and Lachance, 2001; Pande and Akoh, 2010). Regarding protein composition in latex fluid, it is known that lattices of *F. carica* contain multiple forms of proteolytic enzymes (Liener and Friedenson, 1970).

Euphorbiaceae

Euphorbia characias L. (*Euphorbiaceae*) is one of the oldest known medicinal plants of the Western tradition. It is described in most ancient treatises of Greek and Latin medicine (Baumann, 1993), and was held in great esteem up to the development of modern medicine, which made obsolete its use as a powerful cathartic and emetic. *E. characias* is nowadays best known as a garden plant (Appendino et al., 2000). It is one of the most widespread ornamental spurges, and several varieties have been developed, substantially expanding the habitat of this Mediterranean species (Turner, 1995). Two geographical varieties are known, the subsp. *characias* found in the western Mediterranean region, and the subsp. *wulfenii*, which grows in the Balkans, Greece, and Turkey (Turner, 1995). Both subspecies were found active in the mouse ear erythema assay (Evans and Kinghorn 1977). This and the use of the plant as a fish poison (Turner, 1995) suggest the presence of phorbol-type diterpenes.

LATEX LIPASES

Lipases, also known as triacylglycerol ester hydrolases (EC 3.1.1.3), are one of the most versatile biocatalyst with a remarkable ability to achieve a wide range of bioconversion reactions using a variety of substrates. Moreover, lipases possess the unique property of working at a lipid/water interface mainly in organic media (Gupta et al., 2003). In most instances, commercial lipases are generally produced from animals or microorganisms (Kilcawley et al., 2002, Mendes et al., 2012). Nonetheless, most of plant lipases are relatively inexpensive due to their wide availability from natural sources. Plant lipases are mostly found in energy reserve tissues, for example, oilseeds. They act as biocatalysts which are attractive due to their high substrate specificity, low production cost and easy pharmacological acceptance due to their eukaryotic origin. Hence plant lipases represent better potential for commercial applications in organic synthesis, food, detergent and pharmacological industries (Seth et al., 2014). As a result, plant lipases are generally more accepted for food or medicinal applications. However, low expression, uneconomical fold purity and the plethora of difficulties related to their recombinant expression has been limiting their commercial applicability and posing challenges to many researchers (Seth et al., 2014). In addition, the major impediment for its implementation at large scale is the low content of enzyme in the post-germination seeds, bran portion of the grain and wheat gem. Noticeably, *Caricaceae* or *Euphorbiceae* overcome this disadvantage as their enzymes are available in large amounts stored in their latex (Villeneuve, 2003; Paques and Macedo, 2006). In this context, the lipolytic complex of enzymes present in *C. papaya* which, otherwise stated, are referred to as CPL when they are found in the crude latex without pretreatment or pCPL when they are in a crude lipase preparation, hold several advantages over their microbial and animal counterparts such as: (i) good stability to a wide range of pH, temperature, organic solvents and to the presence of other catalysts such as lysozyme, amylase, pectine esterase, thioglucosidase, phosphatase acide, invertase, catalase, peroxidase, lipoxydase,… (Abdelkafi et al., 2011); (ii) relatively inexpensive, e.g. the price is approximately about one third that of crude

Candida rugosa lipase (CRL) (Gandhi et al., 2001; Campillo-Alvarado and Tovar-Miranda, 2013); (iii) can be considered "self-immobilized" enzymes since they are naturally bound to a non-water soluble matrix and thus, do not require further support and can be both recovered and reused (Abdelkafi et al., 2011); (iv) the active sites do not require interfacial activation prompted by detergents such as the pancreatic lipase (Mendes et al., 2012; Giordani et al., 1991); (v) the regio-, stereo-, typo- and substrate selectivities offer high versatility in diverse biochemical reactions; (vi) can be sustainably collected from the industrial papaya agro-waste of sick and unripe fruits (Mendes et al., 2012).

ENZYMATIC (LIPASE) PROPERTIES OF SOME LATEX

The optimum parameters that influence the lipolytic activity of some latex are summaries at Table 1. The lipolytic activities of babaco (Vasconcellea x Heilbornii cv.) and *Plumeria* latex were first measured using sunflower oil as substrate at pH 8 and at temperatures varying from 30 to 70°C by Cambon et al. (2006). Maximum activity was observed at 50°C for babaco (260 IU/g). At 55 and 60°C, significant thermal deactivation was observed for babaco, with 49 and 56% losses of activity, respectively. *Plumeria* appeared to be less sensitive to thermal denaturation and was shown to express its maximum lipolytic activity at 55°C (1400 IU/g), (Table 1). The optimum pH for babaco latex was 7, whereas for *Plumeria* latex, two optimal pH values (4 and 7) were observed. With regard to esterification and acyl transfer reactions, the influence of thermodynamic water activity on reaction yields was determined and correlated with water sorption and desorption isotherms. When babaco latex is used as a biocatalyst, optimal synthesis reaction yields are obtained when the enzymatic extract is stabilized at a water activity (a_w) value of 0.38, which corresponds to a water content of 5.7%. This optimal level of hydration is located on the linear portion of the biocatalyst's sorption isotherm, where the water molecules exhibit high-energy interactions with the protein network (Cambon et al., 2006). In synthesis reactions (esterification, alcoholysis, and interesterification) biocatalyzed by *Plumeria* latex, correlation between best reaction yields and water activity cannot be done. Indeed, the sorption isotherm plot has an atypical shape, indicating that water might be trapped in the latex matrix and, consequently, that the water content of the biocatalyst is highly dependent on the hydration history of the latex (Cambon et al., 2006).

Using tributyrin as substrate, a high level of lipase activity reaching 2,000±185 U/g of CPL was measured using a 10% w/v dispersion of CPL powder in deionized water (Table 1). The lipolytic activity of *C. papaya* latex on the short chain triglyceride tributyrin was described several years ago (Giordani et al., 1991). However, tributyrin is partly soluble in water and some esterases which are active on this substrate did not show any activity on a true lipase substrate such as olive oil. It was observed in a more recent study, however, that CPL could hydrolyze the long chain triglycerides present in test meals and could therefore be considered as a source of true lipase activity (Abdelkafi et al., 2009). It was confirmed here (Table 1) that CPL is active on olive oil (256±8 U/g) as well as on trioctanoin (983±29 U/g). CPL is much more active on short and medium chain TAGs than on long chain TAGs, as occurs with most lipases (Ngando et al., 2006). It is worth noting here that the specific activity of CPL on olive oil was similar to that detected in the dry mesocarp of oil palm fruit (250±14 U/g) (Ngando et al., 2006). CPL was also found to hydrolyze phosphatidylcholine with a specific activity of 65±3 U/g, but showed no activity on cholesterol oleate. Several TAG lipases have been found to show a dual TAG lipase/ phospholipase A1 activity (Simons et al., 1998; Thirstrup et al., 1994) but only if a pure CPL enzyme is obtained it will be possible to determine whether the phospholipase and lipase activities measured with crude CPL are due to the same enzyme.

A a_w affect the reaction rate, enantioselectivity and equilibrium of *C. papaya* lipolytic enzymes, as was reported in the resolution of different (R,S)-2-methylalkanoic acids; CPL showed a maximum initial rate for the (S)-enantiomers (VS) at low water activity (aw = 0.03), whereas the maximum E (enantiomeric ratio, defined as the ratio of initial rates, VS/VR or VR/VS) was achieved by increasing aw to 0.33, further separating VR from VS, although with the penalty of triggering hydrolysis instead of esterification (Chang and Ho, 2011). One of the most advantageous characteristics of the lipolytic enzymes of *C. papaya* is their ability to work efficiently under a broad range of pH and temperature. The good thermostability of the enzymes is attributed to the lipase immobilization in the non-soluble matrix of the latex (Ng and Tsai, 2005). When olive oil was used as the substrate, CPL activity was found to be optimum at pH levels ranging between 9 and 9.5 (Abdelkafi et al., 2001), and the kinetics of fatty acid release were linear for at least 5 min when the pH value was equal to or below 9. At pH values above 9, the kinetics were linear for only 1 to 2 min. These data suggest that CPL is less stable at high pH levels. Optimum conditions for assaying CPL activity on olive oil were therefore set at pH 9. No significant activity could be detected at pH 6 or less (Table 1). The optimum pH range for CPL activity is similar to that determined in the case of other plant lipases from palm oil fruit (Ngando et al., 2006) and babaco (Cambon et al., 2008). When tributyrin and trioctanoin were used as substrates, the maximum activity of CPL was recorded at pH 8 and 9, respectively.

As can be expected, temperature plays a major role in the reaction kinetics; an increase could facilitate the diffusion coefficients of substrates migrating to enzyme-

Table 1. Optimum parameters that influence reaction kinetics of enzymatic properties of some latex and their specificity.

Family	Plants	Optimum parameters				MA[3] (IU/g)	Yield[4] (%)	Alcohols substrates	Type of coordination-site location	Reaction medium	Specificity	Specific activity[a] (IU/g)	Lipids substrates	References
		T[1] (°C)	pH	a_w	WC[2] (%)									
Caricaceae	*C. pentagona* (Babaco)	45 to 50	7	0.38 or < 0.5	5.7	260 to 275	14	Butanol	NA	Solvent-free	*Sn-1,3*	109[e]	Sunflower oil or pure monolein or diolein	Cambon et al., 2006; Chen et al., 2005; Cambon, 2008
Apocynaceae	*P. rubra*	55	4 and 7	0.44	NA	1400	25 to 32	Butanol	NA	Solvent-free	*sn-3*	7400[e]	Sunflower oil or pure monolein or diolein	Cambon et al., 2006; Cambon et al., 2008
Caricaceae	*C. papaya*	50	4 and 10	< 0.11	2	814±38	75	Methanol	Surface	Solvent-free	*Sn-1,3/ sn-3*	256±8[d]	Olive oil[b]	Chang and Ho, 2011; Campillo-Alvarado and Ricardo Tovar-Miranda, 2013; Abdelkafi et al., 2011; Villeneuve et al., 1995; Foglia and Villeneuve, 1997; Villeneuve et al., 1997a; Gonzalo Campillo-Alvarado and Ricardo Tovar-Miranda, 2013; Cambon et al., 2008
												983±29[d]	Trioctanoin[b]	
												2,00±185[d]	Tributyrin[b]	
												65±3[d]	Phosphatidylcoline[c]	
												0	Cholesterol oleate	
Euphorbiaceae	*E. characias*	37 or 50	5 and 8	NA	NA	2909±29	78	Methanol	NA	Mechanically stirred medium of water and oil	*sn-3*	1589±40	Triacetin	Paloccia et al., 2003; Caro et al., 2000; Giordani et al., 1991
						6739±10						3379±2	Tributyrin	
						13369±10						5459±13	Tricaprilin	
						4869±39						3159±5	Linseed oil	
						9259±25						7359±3	Sunflower seed oil	
Euphorbiaceae	*E. wulfenii*	NA	5 and 8	NA	NA	2299±11	80	NA	NA	NA	NA	209±1	Triacetin	Palocci et al., 2003
						7199±1						1379±8	Tributyrin	
						10079±10						1809±13	Tricaprilin	
						3919±21						879±2	Linseed oil	
						4439±40						2509±2	Sunflower seed oil	

[1] temperatures; [2] water content; [3] Maximum activity; [4] % of FFAs released after 1 h; NA: Not available; SA: Specific activity; [a]: Values are means ± SD (n=3); b = Assays with triacylglycerols were performed at 37°C in 2.5 mM Tris-HCl buffer, 150 mM NaCl and at pH 9; c = Assays with phospholipids were performed at 37°C in 7.5 mM $CaCl_2$, 13.3 mM NaTDC and at pH 8; [d]: U/g; [e]: IU/mg of lipases.

active sites, thus enhancing the reaction rate (Varma et al*.,* 2008). This was observed in the increment of the activity of several reactions carried out above room temperature (Tecelão et al., 2012; Lee and Foglia, 2000). A temperature screening of the lipolytic activity on olive oil showed its peak at 50°C, although above 37°C the residual activity of pCPL started to decrease after 1 or 2 min, suggesting that the activity improvement at high temperatures is accompanied by a loss in its stability (Abdelkafi et al., 2011). On the other hand, pCPL was found stable at pH's from 4 to 10, preserving the 75% of its activity after 24 h of incubation at pH 10, whereas short inactivation times were observed out of this range

(Abdelkafi et al., 2011). It is worth noting that only a few microbial lipases, such as that of *Thermomyces lanuginosus* (*Humicola lanuginosa*) (Boel and Huge-Jensen, 1998) have shown similar levels of resistance so far in a large pH range up to pH 10. Concerning lipase activity, the majority of reactions are often carried out in nearly neutral to alkaline pH (Tecelão et al., 2012; Caro et al., 2004; Lee and Foglia, 2000). For example, the CPL mediated hydrolysis in olive oil found its optimum at pH levels between 9 and 9.5 (Abdelkafi et al., 2011), while the SA in the hydrolysis of TAGs present in human diet was better at pH 6 (Abdelkafi et al., 2009). These two pH values account for the observations of (Paques and Macedo, 2006) who by an ammonium sulfate pretreatment enabled the enzyme to work efficiently at pH 6. Alternatively, delipidation with acetone provided an improvement in the lipase activity profile at pH levels close to 9.5 (Paques and Macedo, 2006).

STRUCTURE AND MECHANISM OF THE LIPOLYTIC ENZYMES OF PLANT LATEX LIPASES

Experimental studies have been carried out in order to analyze the ability of lipases to hydrolyse vegetable oils and phosphatides under different conditions (Hara et al., 1997; Mustranta et al., 1995). Marked differences were observed in lipase hydrolytic activity in terms of source, degree of purity, state (free or immobilized), substrate, and reaction medium (solvent-free or biphasic).

A recent screening on latexes of *C. papaya* made it possible to count non-proteic molecular species such as saturated and unsaturated fatty acids, tocopherols, the tocotrienols, alcohols triterpenic, sterols and the possible presence of polyisoprene chains covalently bonded to phospholipid molecules forming a polymeric matrix (Barouh et al., 2010). The proteins quoted above bound once, confers a colloidal stability on latex, which makes the purification of the lipolytic enzymes present in the latex hard to achieve with common separation techniques (Azarkan et al., 2003; Dhouib et al., 2011). According to the literature, only three proteins with lipolytic properties present in the latex of *C. papaya* have been characterized through the aid of the recent sequencing of the *C. papaya* genome (Ming et al., 2008; Campillo-Alvarado and Tovar-Miranda, 2013), although without being purified to homogeneity. Among them GDSL-motif carboxylester hydrolase (CpEst) whose activity was found responsible for the hydrolysis of tributyrin and vinyl esters tested during the analysis. Besides, CpEst did not show a considerable specific activity (SA) towards long chain and medium chain TAGs, in contrast to the whole latex activity, acting as an esterase rather than a true lipase, which strongly suggested that total lipolytic activity in the crude latex could not be attributed to one enzyme (Abdelkafi et al., 2009). Another protein extracted from *C. papaya* is CpLip1 that is also likely to code for the *C. papaya* protein. CpLip1 was identified as a member of the castor bean acid lipase structural family and showed SA towards both short and long TAGs (Dhouib et al., 2011). Results from the literature showed that both CpLip1 and CpEst share a catalytic triad which is similar to that of serine proteases (Pleiss et al., 1998; Brady et al., 1990) - a nucleophilic serine (Ser) residue activated by a hydrogen bond in relay with histidine (His) and aspartate (Asp) in addition to a relatively hydrophilic oxyanion hole that forms hydrogen bonds to the tetrahedral intermediate. However, the active site varies in the amino acids location in the protein (Dodson and Wlodawer, 1998). A major difference between the two enzymes lies on the sequence that forms a “lid” of surface loop that surrounds the catalytic Ser that needs to undergo a conformational change before accessing the whole active domain (Dhouib et al., 2011). On the other hand, the putative structure model of CpEst suggested that the catalytic triad is exposed at the surface of the molecule without a “lid” domain and a binding site for long chain fatty acids (Abdelkafi et al., 2009).

C. papaya lipase (CPL) represents an emerging and versatile biocatalyst (Domínguez de María et al., 2006). This fact is confirmed from the high number of applications described in recent years (Foglia and Villeneuve, 1997a; Mangos et al., 1999; Campillo-Alvarado and Tovar-Miranda, 2013). Its availability as a “natural immobilized” catalyst, combined with a competitive price, makes CPL a promising catalyst in the biotransformations field. In fact, a study of the selectivity of different fatty acid ethyl esters in the CPL-catalysed interesterification of tripalmitin has been reported (Gandhi and Mukherjee, 2000a, b). Interestingly, the use of fatty acid ethyl esters as acyl donors led to a higher CPL selectivity towards the medium-/long-chain derivatives, as well as sn-1 selection. These results seem to be at odds with the other works developed with free fatty acids. A reactant-dependent positional specificity of lipases has been suggested for the explanation (Gandhi and Mukherjee, 2000a, b). Finally, the enzymatic transesterification of tricaprylin with various lauric acid derivatives as acyl donors gave good yields in terms of transesterified triacylglycerols. Vinyl laurate as the best acyl donor (Villeneuve et al., 1997b) presumably resulted from the irreversibility of that reaction, derived from the formation of vinyl alcohol which rapidly tautomerises to acetaldehyde, thus shifting the enzymatic reaction toward the products formation (Weber et al., 1997). Currently, applications regarding fats and oils modification, esterification in organic media, and asymmetric resolution of several chiral acids, as well as non-natural α-amino acids, have been also reported (Mukherjee and Kiewitt, 1996; Borgdorf and Warwel, 1999; Gandhi and Mukherjee, 2000a; Villeneuve et al., 2005).

Like *C. papaya* lipase a study on the catalytic properties of frangipani (*P. rubra*) latex lipase revealed that this latter lipase has a high capacity to catalyze fatty

acid esterification in solvent-free medium in less than an hour, with over 90% yield (Cambon et al*.,* 2006). Due to this capacity, this lipase could be used in a two-step biodiesel production process (hydrolysis and esterification) in association with a second lipase, such as that extracted from *Jatropha curcas*, that is highly active in TAG hydrolysis. Sousaa et al*.* (2010) recently showed that lipase from germinated *J. curcas* seeds could be used for TAG hydrolysis in a hydroesterification process, with 98% yields achieved after 2 h of reaction, without specificity with respect to the fat source used. Moreover, the catalytic activity of the lipase in crude babaco (*C. pentagona*) latex has been studied in transesterification and esterification reactions (Cambon et al., 2009; Dhuique-Mayer et al*.,* 2003). Cambon et al*.* (2009) showed that babaco lipase has catalytic activity during alcoholysis of sunflower seed oil with highly excessive amounts of various primary alcohols in a solvent-free system. Despite its sensitivity to short-chain alcohols such as methanol, the stepwise addition method curbs the inhibitory effect of methanol and enables transesterification yields of around 70% at 30°C after 15 h (Shimada et al*.*, 1999; Shimada et al., 2002).

Preliminary screening on *Euphorbia* species latex showed high lipolytic activity in *E. wulfenii*. For both *E. characias* and *E. wulfenii* latex it was find high lipolytic activity toward medium and long acylic chain triglycerides, but no hydrolytic activity on monoesters and phospholipids was detected (Palocci et al*.,* 2003). Moreover, no synthetic activity was pointed out using as substrates natural and endogenous terpenols (Palocci et al., 2003). The presence of esterase activity in latex of two *Euphorbia* species (*E. pulcherrima* and *E. lathirys*) was also described (Warnaar, 1987) suggesting that, *in vivo*, this activity could be involved in the hydrolysis reaction of triterpenol esters and the subsequent storage of free triterpenols inside the lipidic particles of latex. However, Palocci et al*.* (2003) demonstrated that such enzymes cannot be related to the terpenic metabolism. In fact the enzymes responsible for lipolytic activity in the latex of *E. characias* and *wulfenii* described in the work of Palocci et al*.* (2003) are not able to hydrolyse monoester or to synthesise terpenol esters starting from natural and endogenous terpenols. Moreover, in agreement with Warnaar's hypothesis (Warnaar, 1987), the relative terpenol ratio was constant during the biological cycle and the esterified terpenol fraction was present in negligible quantities for both species studied. On this basis lipolytic activity recovered in latex seems to be due to ''true lipases'' (Huang Anthony, 1984) acting on triglycerides probably present in latex (Hasma and Subramanian, 1986).

PLANT LATEX LIPASES PURIFICATION

Lipases have been purified from various plant parts (Seth et al., 2014). Many plant parts such as leaves of *Triticum* L. species (Kharazian et al., 2009), whole plant parts of *Ricinus communis* (Shahwar et al., 2010), oat bran, etc., are rich in phenolic content. This makes the purification step laborious and the yield is also very low when seed, leaf or latex is used for direct extraction. Plant seeds also contain high amount of lipids which is another associated major problem in plant lipase purification as such lipids interfere in SDS–PAGE. The consequence is a smeared discontinuous gel. Therefore, delipidation step becomes compulsory for extracting plant lipase prior to any other purification steps. This adds to the production cost and time-consuming.

As reported by Seth et al. (2014) a few exception most of the purification involves chromatography techniques. It is also visible that the yield is very low. Ben-Hamida and Mazliak (1985) reported that some of the traditional procedures such as clarification, precipitation, ultrafiltration, differential and density gradient centrifugations results in a low final yield of purified plant lipase. Alternatively, ion exchange and gel filtration chromatography used for purification of plant lipases results in good yield. Moreover, Lazreg-Aref et al. (2012) are recently purified lipase to homogeneity from *F. carica* L. latex of the Zidi variety from *Moraceae* family using silica gel chromatography (Table 2).

BIODIESEL PRODUCTION

The frequent and future scarcity of fossil fuels, combined with concerns over the consequences of dependency on this type of energy source, in terms of changes in the Earth's climate, has forced the world to find alternatives that are less harmful to the environment (de Araújo et al*.*, 2013). Renewable energy sources, especially vegetable fuel, have appeared as an important alternative (Santana et al*.*, 2010).

Biodiesel is made from renewable biological sources and it does not produce sulfur oxide and may reduce soot discharge by one third that of existing petroleum-based products (Ranganathan et al*.*, 2008). Biodiesel in industrial applications may be produced by chemical-catalyzed or enzyme-catalyzed methods. The biodiesel produced by chemical catalyst has several drawbacks such as difficulty in removal of acid or base catalysts from product, high energy requirements, difficulties in the recovery of the catalyst and potential pollution to the environment (Winayanuwattikun et al*.*, 2011; Tan et al*.*, 2010). Enzyme-catalyzed biodiesel production has received more attention because of its advantages, such as low energy consumption, mild operating conditions, nontoxicity, and environment friendly processes, as compared with the chemical-catalyzed method (Dwiarti et al*.*, 2010; Lee et al*.*, 2011). However, the enzyme-catalyzed method is not favored for industrial use because the high cost and low stability of lipases limit its potential application (Chen and Wu, 2003; Soumanou and Bornscheuer, 2003).

Table 2. Purification strategies for plant latex lipases.

Family	Plant sources	Purification steps	Fold increase/ yield	References
Euphorbiaceae	*E. characias*	Acetone/H_2O and silica column	NA	Palocci et al., 2003; Padiglia et al., 1998
Euphorbiaceae	E. *Wulfenii*	Acetone/H_2O and silica column	NA	Palocci et al., 2003; Padiglia et al., 1998
Moraceae	*Ficus carica L.*	Silica gel chromatography	8.5-fold/68.5%	Lazreg-Aref et al., 2012
Caricaceae	*Carica papaya*	Extraction in aqueous two-phase system	NA	Nitsawang et al., 2006
Caricaceae	Babaco or *Carica pentagona (Vasconcellea* x *Heilbornii Cv.)*	Extraction in aqueous two-phase system	15-fold/ NA	Chen et al., 2005
Apocynaceae	*Plumeria rubra*	Hexane/Steric exclusion chromatography	NA	Cambon, 2008

NA: Not available. Fold increase is the ratio of specific activity of the final purified product to the initial specific activity; and yield is the ratio of initial enzyme titer to the final titer obtained after the purification process.

Catalytic conversion techniques for transesterification

Alkalis used for transesterification of oil include NaOH, KOH, carbonates, and alkoxides such as sodium methoxide, sodium ethoxide, sodium propoxide, and sodium butoxide. Alkali-catalyzed transesterification proceeds approximately 4000 times faster than that catalyzed by the same amount of an acidic catalyst (Formo, 1954; de Araújo et al., 2013), and is thus most often used commercially. Potassium hydroxide (KOH) and sodium hydroxide (NaOH) are high sensitive to the purity of the reaction being affected by the water and free fatty acids contents (Marchetti et al., 2007). The presence of water may cause the ester saponification under alkaline conditions. Thus, the glycerides and alcohol must be substantially anhydrous because water causes a partial reaction change to saponification, which produces soap (Wright et al., 1944). Moreover, the free fatty acids can also react with the alkaline catalyst producing soaps and water. The saponification does not only use up the catalyst, but also causes the formation of emulsions which impair the biodiesel separation, recuperation and purification. Therefore, dehydrated vegetable oil with free fatty acids content lower than 1%, anhydrous catalysts and anhydrous alcohol are essential for the commercial feasibility of alkaline catalyst systems (Enweremadu and Mbarawa, 2009). Ma et al. (1998) suggested that the free fatty acid content of the refined oil should be as low as possible, below 0.5%, and Feuge and Grose (1949) also stressed the importance of oils being dry and free of free fatty acids. Freedman et al. (1984) reported that ester yields were significantly reduced if the reactants did not meet these requirements; sodium hydroxide or sodium metboxide reacted with moisture and carbon dioxide in the air, diminishing their effectiveness.

Studies report that acid catalysts are insensitive to the acidity value and are better than alkaline catalysts for vegetable oils with acidity value higher than 1% (Freedman et al., 1984).

Acids used for transesterification include sulfuric, phosphoric, hydrochloric, and organic sulfonic acids. Although transesterification by acid catalysis is much slower than that by alkali catalysis (Freedman et al., 1984; Ma and Hanna 1999; Srivastava and Prasad, 2000), acid-catalyzed transesterification is more suitable for glycerides that have relatively high free fatty acid contents and more water (Freedman et al., 1984; Aksoy et al., 1988). Aksoy et al. (1988) reported that it was necessary to perform transesterification under an acidic condition when the oil component was a low grade material such as sulphur olive oil. In general, the ethyl esters of monounsaturated or short-chain fatty acids with 2% sulfuric acid should make good alternative fuels (Klopfenstein and Walker, 1983). The transesterification by acid catalysis starts by mixing the oil directly with acidified alcohol, in a way that separation and transesterification can occur in a single step, being alcohol the esterification solvent and reagent (Cervero et al., 2008).

Researches on biodiesel have focused on the use of solid acid catalysts known as heterogeneous catalysts. Sulfonic resins, such as Nafion NR50, sulfated zirconia and tungstated zirconia may catalyze transesterification reactions as effectively as sulfuric acid (de Araújo et al., 2013). Studies report that the solid acid catalyst ideal to the transesterification of used cooking oil is expected to have features such as interconnected system of large pores, moderate and high concentrations of strong acids sites and hydrophobic surface (Lotero et al., 2005).

The advantages of using solid acids catalysts are insensitivity to acidity value; esterification and

transesterification may be carried out simultaneously; the catalyst is easy to be recovered; water washing biodiesel is unnecessary; generally high performance in esters; much lower catalyst requirements per tons of biodiesel produced than in other processes; and catalysts may be used for a longer period of time and are environmentally friendly. However, these systems operate under high temperature and pressure.

Although chemical transesterification using an alkali-catalysis process gives high conversion levels of triglycerides to their corresponding methyl esters in short reaction times, the reaction has several drawbacks: it is energy intensive, recovery of glycerol is difficult, the acidic or alkaline catalyst has to be removed from the product, alkaline wastewater requires treatment, and free fatty acids and water interfere with the reaction (Table 4).

Lipases are also able to effectively catalyze the transesteritication of triglycerides in either aqueous or nonaqueous systems, and as shown in Table 4, enzymatic tranesterification methods can overcome the problems mentioned above. In particular, it should be noted that the by-product, glycerol, can be easily recovered without any complex process, and also that free fatty acids contained in waste oils and fats can be completely converted to methyl esters. On the other hand, in general the production cost of a lipase catalyst is significantly greater than that of an alkaline one (Wu et al., 1999).

Moreover, the following advantages for the use of lipases can be mentioned (Marchetti et al., 2007).

i) Possibility of regeneration and reuse of immobilized waste, as it can be left in the reactor if reactivity is kept low.
ii) Higher enzyme thermal stability due to its inactive state.
iii) Easier separation from the product.

Some disadvantages include,

i) Loss of some initial activity due to the volume of oil molecule.
ii) Number of support enzymes is not uniform.
iii) Biocatalysts are more expensive than natural enzymes.

Non-catalytic conversion techniques for transesterification

To overcome delays in the initial reaction time caused by the low solubility of alcohol in the triglyceride phase the non-catalytic options are designed. A common approach consists in the use of a solvent soluble in methanol and oil. The result is a fast reaction, on the order of 5 to 10 min with no catalyst residues, in any phase. One of these cosolvents is the tetrahydrofuran, chosen, partially, due to its boiling point near that of methanol and the need of a very low operational temperature, around 301°C.

A second approach was developed by Saka and Kusdiana (Saka and Kusdiana, 2001) who made a fundamental study of biodiesel production in supercritical methanol. They demonstrated that preheating to a temperature of 350°C and treatment for 240 s in supercritical methanol was sufficient to convert rapeseed oil to methyl esters. Moreover, while the methyl esters produced were basically the same as those obtained in the conventional method with a basic catalyst, the methyl ester yield of the supercritical methanol method was higher. Kinetic analyses of the reactions in subcritical and supercritical methanol revealed that the rate of rapeseed oil conversion to methyl esters increased dramatically in the supercritical state. A reaction temperature of 350°C and a molar ratio of methanol to rapeseed oil of 42 to 1 were considered to be the best conditions (Kusdiana and Saka, 2001). Some advantages of its application are (Balat and Balat, 2008):

i) Glycerides and free fatty acids react with equivalent rates.
ii) The homogeneous phase eliminates diffusive problems.
iii) The process tolerates great percentages of water in the catalytic process of the feedstock requiring periodical removal of water or an intermediary phase to prevent catalyst deactivation.
iv) The catalyst removal phase is eliminated.
v) If a high methanol/oil ratio is used, the total oil conversion can be achieved in few minutes.

Despite all the above mentioned advantages, the supercritical methanol method has serious disadvantages, such as:

i) The process operates at extremely high pressures (25 to 40 MPa);
ii) The high temperatures (350 to 4001°C) result proportionally in high heating and cooling costs;
iii) High methanol: oil ratios (generally established at 42:1) involve high costs for the evaporation of the unreacted methanol.

POTENTIAL LATEX FOR BIODIESEL IN BENIN

Since last few years many laticifers plants have been identified all over the world and in Benin. These plants are distributed on all extent of the Benin territory. They often belong to the family of *Apocynaceae*, *Asclepiadaceae*, *Euphorbiaceae* or *Moraceae* (Table 3). Hence, focus needs to be shifted to lipases plants available in Benin and the details of such potential lipase plant are shown in Table 3. The listed species are used at various ends. They constitute a food, medicinal source as well that of wood for the populations (Table 3). The vegetable near total of the species of cover of Benin is used in traditional medicine by the local populations to fight against diseases (Agbahungba et al., 2001). Various

Table 3. Potential plant latex lipases in Benin.

Family	Botanical name	Common name	Distribution	Latex color	Measured parameters	Use	Part used	References
	Alstonia congensis	Afatin	Sèmè – Dangbo	White	Extraction of proteins; Acute toxicity test; Subacute test; Search for polyphenols by the reaction to ferric chloride; Search for alkaloids ; Search for flavonoïdes by the reaction to the cyanidine; Search for tanins (tanins cathechic by the reagent of STIASNY; tanins gallic); Search for substances quinoniques free and combined by the reaction of BORNTRAEGER; Search for polyterpenes and stéroïdes by the reaction of Libermann-buchard; Search for saponosides; Description of the macromolecules (Search for proteins by the method of LOWRY; Search for polysaccharides)	Treatment of diabetes, interior of plywood, lathed panel, packing-box factory, matches, light boats, moulding, piece of furniture running or elements, interior wood finishing, pencils	Bark, leaf	Ogbonnia et al., 2008; Fofana, 2004
Apocynaceae	*Holarrhena floribunda*	lètin wiwi ou akoyixè ou lengbagbé.	Calavi – Bohicon	White	Toxicity study; Crude protein, fat, crude fiber and total ash contents in the dried leaves were determined using the methods described by Association of Official Analytical Chemists (AOAC, 1990). Carbohydrate (nitrogen free extract) was determined by difference; that is, the sum of the crude protein, fat, crude fibre and total ash deducted from 100. Phytochemical analysis (Mayer, Dragendoff, Wagner and picric reagents were used to test for Alkaloid. Frothing test for saponin, ferric chloride test for tannin while Salkowski test for cardiac glycosides) DPPH radical assay; Lipid peroxidation and thiobarbituric acid reactions; Hydroxyl radical scavenging assay; Nitric oxide radical inhibition activity; Determination of total antioxidant capacity; Determination of total phenol; Reducing power	Veterinary healers, antioxidant, anti-dysenteric, diuretic and febrifuge, intestinal parasitoses, the ascite and sterility	Bark, leaves and roots	Tamboura et al., 2005; Badmus et al., 2010 ; Medecine douce - Medecine africaine, 2014
	Rauvolfia vomitoria	lètin, klanklan tin.	Calavi – Bohicon	White	Acute oral toxicity test; Phytochemical test; Phytochemical screening	Anticonvulsant, insomnia, depression and madness.	Leafs and roots	Amole et al., 2009 ; Medecine douce - Medecine africaine, 2014
	Saba comorensis	NA	Bassila	White	NA	Food, traditional medicine, oedema generalized	Fruit and Leafs	Olivier et al., 2012
	Thevetia peruviana	Tantohu (Fon) ; Olomiojo (Yoruba et Nagot) ; Batonè (Bariba),	Calavi	White	NA	Medicinal plant, laxative, emetic, look after the intermittent fevers	Bark, Leafs	Arbonnier, 2002, Schmelzer and Gurib-Fakim, 2006
Euphorbiaceae	*Anthostema aubryanum*	NA	Sakété	White	Diversity of the woody settlement of a dense forest in sub-wet zone; Cartography and floristic characterization of the marshy forest	Latex = poison; strong vermifuge activity counters the larvae of *Haemonchus contortus* in vitro;	Latex; bark; stem	Hecketsweiler, 1991; Nkeoua and Boundzanga, 1999; Adjakpa et al., 2011
	Euphorbia heterophylla	NA	Calavi	White	Chromatography (The crude methanolic and aqueous extracts were subjected to phytochemical screening); Anti-inflammatory activity	Medicinal use; treatment of constipation, bronchitis and asthma	Leaves; fruits; flowers	Falodun et al., 2004; Falodun et al., 2003; Falodun et al., 2006

Table 3. Contd.

	Milicia excelsa	Iroko/Lokotin	Calavi – Bohicon	White	NA	Exterior wood finishing: Parquet floor Shipbuilding (bordered and bridge);Cabinet work (piece of furniture of luxury); Interior wood finishing: Distinct plating; Skirting; Light frame		CIRAD, 2011
Sapotaceae	*Vitellania poradoxa*	NA	Dan	White	NA	Food, medicinal and cosmetic	Fruits, bark	Medecine douce - Medecine africaine 2014
	Manilkara multinervis	NA	Natitingou	White	NA	Piles in the construction of the houses on pile, Clothes industry of the frame of the houses and the attics	Wood	Medecine douce - Medecine africaine 2014; Agbahungba et al., 1998

Table 4. Comparison between alkali-catalysis and lipase-catalysis methods for biodiesel fuel production (Fukuda et al., 2001; Mounguengui et al., 2013).

Composition	Alkali-catalysis process	Lipase-catalysis process
Reaction temperature	60-70°C	30-40°C
Free fatty acids in raw materials	Saponified products	Methyl esters
Water in raw materials	Interference with the reaction	No influence
Yield of methyl esters	Normal	Higher
Recovery of glycerol	Difficult	Easy
Purification of methyl esters	Repeated washing	None
Production cost of catalyst	Cheap	Relatively expensive

bodies of the plants are used for this purpose to know: sheets, fruits, flowers, barks and roots (Azonkponon, 2001). 92.86% of the species are used as wood energy. With the exception of the species taboos which vary according to the various ethnos groups, all the species of trees are used as and charcoal firewood. 39.29% are used as edible plants (Houngnon, 1981; Sokpon and Lejoly, 1996), 35.21% like work wood, 17.87% as service wood (return in this category all wood which are used in the clothes industry of the frame of the houses and the attics) and 3.57% in local arts and crafts (Dossou et al., 2012).

Conclusion

Plants produce a diverse range of bioactive molecules, making them rich source of different types of bio-catalyst. It is the case of lipases which are now widely used in various industry sectors such as in detergency applications, fatty wastes treatments, pharmaceutical syntheses or oils and fats modifications.

The lipolytic enzymes of laticifers plants have demonstrated to be versatile biocatalysts with the ability to discriminate a wide number of substrates from various applications. In addition, this lipase aggregate holds important advantages over other microbial, animal and plant lipases being its sustainable availability from agro-waste, its "self-immobilized" nature which conferring the ability to work under a broad range of environments and its easy pretreatments being the most remarkable features. Notwithstanding the enormous potential of the lipases present in laticifers plants, the lack of experimentation carried out at the industrial scale prevents its implementation in various bioprocesses, such as the production of high value lipids with improved properties, the manufacture of medical articles and biodiesel engineering.

Till date, a very large majority of lipases that are used in these processes are obtained from microbial sources. Comparatively, the use of plant lipases is much less developed. However, plant enzymes can be also envisaged as biocatalysts for lipids bioconversions. Especially, high activities in hydrolysis and synthesis reactions have been found in some laticifer plants like *C. papaya* and *E. characias*. Concerning the former, favourable applications in the synthesis of low-calorie TAGs,

medium chain TAGs or for the production of conjugated linoleic acids enriched TAGs were reported. Among the *Caricaceae* family, it was shown recently that the unripe fruit of the babaco plant (*Vasconcellea* x *heilbornii*; ex. *C. pentagona*), native to the subtropical mountains of Equator, contains a latex similar to that in *C. papaya*. This latex also displays a strong lipolytic activity which was characterized in terms of biocatalytic activity and selectivity.

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Conflict of Interests

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REFERENCES

Abdelkafi S, Abousalham A, Fendri I, Ogata H, Barouh N, Fouquet B, Scheirlinckx F, Villeneuve P, Carrière F (2012). Identification of a new phospholipase D in *Carica papaya* latex. Gene 499:243-249.

Abdelkafi S, Barouh N, Fouquet B, Fendri I, Pina M, Scheirlinckx F, Villeneuve P, Carrière F (2011). *Carica papaya* Lipase: A naturally immobilized enzyme with interesting biochemical properties. Plant Foods Hum. Nutr. 66:34-40.

Abdelkafi S, Fouquet B, Barouh N, Durner S, Pina M, Scheirlinckx F, Villeneuve P, Carrière F (2009). In vitro comparisons between Carica papaya and pancreatic lipases during test meal lipolysis: Potential use of CPL in enzyme replacement therapy. Food Chem. 115:100-106.

Abdelkafi S, Ogata H, Barouh N, Fouquet B, Lebrun R, Pina M, Scheirlinckx F, Villeneuve P, Carrière F (2009). Identification and biochemical characterization of a GDSL-motif carboxylester hydrolase from *Carica papaya* latex. Biochim. Biophys. Acta Mol. Cell Biol. Lipids 1791:1048-1056.

Adjakpa JB, Dassoundo HA, Yedomonhan H, Weesie PDM, Akpo E. L (2011). Diversité du peuplement ligneux d'une forêt dense en zone sub-humide : Cas de la forêt de Sakété dans le sud-Bénin en Afrique de l'Ouest. Int. J. Biol. Chem. Sci. 5(6):2291-2305.

Agbahungba G, Sokpon Nm, Gaoué OG (2001). Situation des Ressources Génétiques Forestières du Bénin. L'Atelier sous-régional FAO/IPGRI/ICRAF sur la conservation, la gestion, l'utilisation durable et la mise en valeur des ressources génétiques forestières de la zone sahélienne (Ouagadougou, 22-24 Sept. 1998). Note Thématique sur les Ressources Génétiques Forestières. Une co-publication de la FAO, IPGRI/SAFORGEN, DFSC et ICRAF:36p.

Akoh CC, Chang SW, Lee GC, Shaw JF (2007). Enzymatic approach to biodiesel production. J. Agric. Food Chem. 55:8995-9005.

Aksoy HA, Kahraman, I, Karaosmanoglu F, Civelekoglu, H (1988). Evaluation of Turkish sulphur olive oil as an alternative diesel fuel. J. Am. Oil Chem. Soc. 65:936-938.

Alonso FOM, Oliveria EBL, Dellamora-Ortiz GM, Pereira-Meirelles FV (2005). Improvement of Lipase Production at Different Stirring Speeds and Oxygen Levels. Braz. J. Chem. Eng. 22:1-9.

American Society of Testing Materials Standard (2009). D6751-09a standard speci- fication for biodiesel fuel blend stock (B100) for middle distillate fuel. West Conshohocken, PA: ASTM International. http://dx.doi.org/10.1520/ D6751-09.

Amole OO, Yemitan OK, Oshikoya KA (2009). Anticonvulsant activity of Rauvolfia Vomitoria (Afzel). Afr. J. Pharm. Pharmacol. 3(6):319-322.

Appendino G, Belloro E, Tron GC, Jakupovic J, Ballero M (2000). Polycyclic diterpenoids from *Euphorbia characias*. Fitoterapia 71:134-142.

Azarkan M, El Moussaoui A, van Wuytswinkel D, Dehon G, Looze YJ (2003). Fractionation and purification of the enzymes stored in the latex of *Carica papaya*. Chromatogr. B 790:229-238.

Azonkponon N (2001). Conservation in situ de l'iroko (Milicia excelsa) dans l'aire culturelle Vodun au Bénin. Mem. de DESS (3è cycle) en Aménagement et Gestion des Ressources Naturelles, FSA/UNB, Benin 107 p.

Badmus JA, Odunola, OA, Obuotor EM, Oyedapo OO (2010). Phytochemicals and *in vitro* antioxidant potentials of defatted methanolic extract of *Holarrhena floribunda* leaves. Afr. J. Biotechnol. 3:340-346.

Balat M, Balat H (2008). A critical review of bio-diesel as a vehicular fuel. Energy Convers. Manage. 49:2727-41.

Barouh N, Abdelkafi S, Fouquet B, Pina M, Scheirlinckx F, Carrière F Villeneuve P (2010). Neutral lipid characterization of non-water-soluble fractions of *Carica papaya* latex. J. Am. Oil Chem. Soc. 87:987-995.

Boel E, Huge-Jensen B (1998). Recombinant Humicola lipase and process for production of recombinant (*Humicola* lipase). In: European Patent Applied EP 030521600216.

Borgdorf R, Warwel S (1999). Substrate selectivity of various lipases in the esterification of cis- and trans-9-octadecenoic acid. Appl. Microbiol. Biotechnol. 51:480-485.

Brady L, Brzozowski AM, Derewenda ZS, Dodson E, Dodson G, Tolley S, Turkenburg JP, Christiansen L, Huge-Jensen B, Norskov L (1990). A serine protease triad forms the catalytic centre of a triacylglycerol lipase. Nature 343:767-770.

Brunschwig C, Moussavou W, Blin J (2011). Use of bioethanol for biodiesel production. Progress in Energy and Combustion Science; 38:283e301.

Cambon E (2008). Plantes laticifères : Mise en évidence et applications des activités lipasiques de *Carica pentagona* et *Plumeria rubra*. These pour obtenir le grade de docteur de l'Universite Montpellier II École Doctorale : Sciences des procédés – Sciences des aliments Discipline : Biochimie et technologie alimentaire; 157pp.

Cambon E, Bourlieu C, Salum TFC, Piombo G, Dubreucq E, Villeneuve P (2009). Ability of Vasconcelle x heilbornii lipase to catalyse the synthesis of alkyl esters from vegetable oils. Process Biochem. 44:1265-1269.

Cambon E, Gouzou F, Pina M, Barea B, Barouh N, Lago R, Ruales J, Tsai SW, Villeneuve P (2006). Comparison of the lipase activity in hydrolysis and acyltranfer reactions of two latex plant extracts from Babaco (*Vasconcellea* x heilbornii cv.) and *Plumeria rubra*: Effect of the aqueous microenvironment. J. Agric. Food Chem. 54:2726-2731.

Cambon E, Rodriguez JA, Pina M, Arondel V, Carrière F, Turon F, Ruales J, Villeneuve P (2008). Characterization of typo-, regio-, and stereo-selectivities of babaco latex lipase in aqueous and organic media. Biotechnol. Lett. 30:769-774.

Campillo-Alvarado G, Tovar-Miranda R (2013). Recent advances and applications of the lipolytic activity of *Carica papaya* latex. J. Mol. Catal. B Enzym. 90:49-60.

Caro Y, Villeneuve P, Pina M, Reynes M, Graille J (2000). Investigation of crude latex from various *Carica papaya* varieties for lipid bioconversions. J. Am. Oil Chem. Soc. 77:891-901.

Caro Y, Turon F, Villeneuve P, Pina M, Graille J (2004). Enzymatic synthesis of medium-chain triacylglycerols by alcoholysis and interesterification of copra oil using a crude papain lipase preparation. Eur. J. Lipid Sci. Technol. 106:503-512.

Castro HF, Oliveira PC, Pereira EB (2000). Influence of Substrate Partition Coefficient on the Performance of Lipase Catalyzed Synthesis of Citronellyl Acetate by Alcoholysis. Braz. J. Chem. Eng. 17:4-7.

Cervero PJM, Coca J, Luque S (2008). Production of biodiesel from vegetable oils. Grasas y Aceites. 9(1):76-83.

Chang CS, Ho SC (2011). Enantioselective esterification of (R, S)-2-methylalkanoic acid with *Carica papaya* lipase in organic solvents. Biotechnol. Lett. 33:2247-2253.

Chen CC, Tsai SW, Villeneuve P (2005). Enantioselective hydrolysis of (R, S)naproxen 2,2,2-trifluoroethyl ester in water-saturated solvents

via lipases from *Carica pentagona* Heilborn and *Carica papaya*. J. Mol. Catal. B Enzym. 34:51-7.
Chen JW, Wu WT (2003). Regeneration of immobilized *Candida antarctica* lipase for transesterification. Biosci. Bioeng. 95:466-469.
Cheng YC, Tsai SW (2004). Enantioselective esterification of (RS)-2-(4-chlorophenoxy) propionic acid via *Carica papaya* lipase in organic solvents. Tetrahedron Asymm. 15:2917-2920.
de Araújo CDM, de Andrade CC, de Souza ES, Dupas FA (2013). Biodiesel production from used cooking oil: A review. Renew. Sustain. Energy Rev. 27:445-452.
de Castro HF, Mendes AA, dos Santos JC (2004). Modificação De Óleos E Gorduras Por Biotransformação. Quim. Nova. 27:146-156.
Dhouib R, Laroche-Traineau J, Shaha R, Lapaillerie D, Solier E, Rualès J, Pina M, Villeneuve P, Carrière F, Bonneu M, Arondel V (2011). Identification of a putative triacylglycerol lipase from papaya latex by functional proteomics. FEBS J. 278:97-110.
Dhuique-Mayer C, Caro Y, Pina M, Ruales J, Dornier M, Graille J (2001). Biocatalytic properties of lipase in crude latex from babaco fruit (*Carica pentagona*). Biotechnol. Lett. 23:1021-1024.
Dhuique-Mayer C, Villareal L, Caro Y, Ruales J, Villeneuve P, Pina M (2003). Lipase activity in alcoholysis and esterification reactions of crude latex from babaco fruit (*Carica pentagona*). Oléagineux Corps gras Lipides 10:232-234.
Dodson G, Wlodawer A (1998). Catalytic triads and their relatives. Trends Biochem. Sci. 23:347-352.
Dossou ME, Houessou GL, Lougbégnon OT, Tenté AHB, Codjia JTC (2012). Etude ethnobotanique des ressources forestières ligneuses de la forêt marécageuse d'Agonvè et terroirs connexes au Bénin. Tropicultura 30(1):41-48.
Dwiarti L, Ali E, Park EY (2010). Enhancement of lipase catalyzed-fatty acid methyl esters production from waste activated bleaching earth by nullification of lipase inhibitors. Bioresour. Technol. 101:14-20.
El Moussaoui A, Nijs M, Paul C, Wintjens R, Vincentelli J, Azarkan M, Looze Y (2001). Revisiting the enzymes stored in the laticifers of *Carica papaya* in the context of their possible participation in the plant defence mechanism. Cell Mol. Life Sci. 58:556-570.
Enweremadu CC, Mbarawa MM (2009). Technical aspects of production and analysis of biodiesel from used cooking oil-A review. Renew. Sustain. Energy Rev. 13(9):2205-2224.
Evans F, Kinghorn AD (1977). A comparative phytochemical study of the diterpenes of some species of the genera Euphorbia and Elaeophorbia (*Euphorbiaceae*). Bot. J. Linn. Soc. 74(1):23-35.
European Committee for S. Fat and Oil Derivatives. Fatty acid methyl esters (FAME) (2003). In Determination of Oxidative stability (Accelerated oxidation Test). Brussels, Belgium: European Committee for Standardization (CEN). EN 14112: 2003.
Fahn A (Ed.) (1982). Plant Anatomy, Pergamon Press, London. pp. 134-151.
Falodun A, Agbakwuru EOP (2004). Phytochemical analysis and laxative activity of the leaf extracts of *Euphobia heterophylla* linn (euphorbiaceae). Pak. J. Sci. Res. 47(5):345-348.
Falodun A, Agbakwuru EOP, Ukoh GC (2003). Antibacterial Activity of *Euphorbia heterophylla* Linn (Family *Euphorbiaceae*). Pak. J. Sci. Res. 46(6):471-472.
Falodun A, Okunrobo LO, Uzoamaka N (2006). Phytochemical screening and anti-inflammatory evaluation of methanolic and aqueous extracts of *Euphorbia heterophylla Linn* (*Euphorbiaceae*). Afr. J. Biotechnol. 5(6):529-531.
Feuge RO, Grose T (1949). Modification of vegetable oils. VII. Alkali catalyzed interesterilication of peanut oil with ethanol. J. Am. Oil Chem. Soc. 26:97-102.
Fiorillo F, Palocci C, Soro S, Pasqua G (2007). Latex lipase of *Euphorbia characias* L.: An aspecific acylhydrolase with several isoforms. Plant Sci. 172:722-727.
Fjerbaek L, Christensen KV, Norddahl B (2009). A review of the current state of biodiesel production using enzymatic transesterification. Biotechnol. Bioeng. 102(5):1298-1315
Fofana S (2004). Exploration biochimique sur le pouvoir immunogene de trois plantes en côte d'ivoire: Alstonia boonei (*apocynaceae*), Mitragyna ciliata (*rubiaceae*) et terminalia catappa (*combretaceae*). Faculte de Medecine de Pharmacie et D'odontostomatologie. République du Mali. Thèse de doctorat. P 123.
Foglia TA, Villeneuve P (1997). *Carica papaya* latex-catalyzed synthesis of structured triacylglycerols. J. Am. Oil Chem. Soc. 11:1447-1450.
Formo MW (1954). Ester reactions of fatty materials. J. Am. Oil Chem. Soc. 31:548-559.
Freedman B, Pryde EH, Mounts TL (1984). Variables affecting the yields of fatty esters from transesterified vegetable oils. J. Am. Oil Chem. Soc. 61(10):1638-1643.
Freedman, B, Pryde EH, Mounts TL (1984). Variables affecting the yields of fatty esters from transesteritied vegetable oils. J. Am. Oil Chem. Soc. 61:1638-1643.
Fukuda H, Kondo A, Noda H (2001). Biodiesel Fuel Production by Transesterification of Oils. J. Biosci. Bioeng. 92:405-416.
Gandhi NN, Mukherjee KD (2000a). Specificity of papaya lipase in esterification with respect to the chemical structure of substrates. J Agric. Food Chem. 48:566-570.
Gandhi NN, Mukherjee KD (2000b). Papaya (*Carica papaya*) lipase with some distinct acyl and alkyl specificities as compared with microbial lipases. Biochem. Soc. Trans. 28:977-978.
Gandhi NN, Mukherjee KD (2001). Specificity of papaya lipase in esterification of aliphatic alcohols: A comparison with microbial lipases. J. Am. Oil Chem. Soc. 78:161-165.
Giordani R, Moulin A, Verger R (1991). Tributyroylglycerol hydrolase activity in *Carica papaya* and other lattices. Phytochemistry 30:1069-1072.
Graboski MS, McCormick RL (1998). Combustion of fat and vegetable oil derived fuels in diesel engines. Prog. Energy Combust. Sci. 24:125-164.
Gupta R, Rathi P, Gupta N, Bradoo S (2003). Lipase assays for conventional and molecular screening: An overview. Biotechnol. Appl. Biochem. 37:63-71.
Hara F, Nakashima T, Fukuda H (1997). Comparative study of commercially available lipases in hydrolysis reaction of phosphatidylcholine. J. Am. Oil Chem. Soc. 74(9):1129-1132.
Hasma H, Subramanian A (1986). Composition of lipids in latex of *Hevea brasiliensis* clone RRIM 501. J. Nat. Rubber Res. 1:30-40.
Houngnon P (1981). Espèces forestières à produits comestibles. Tome 1. Enseignement général, Sciences, Sport. Service 22. GTZ. 59 p.
Huang Anthony HC (1984). Plant lipases, in: B. Borgstrom, H.L. Brockman (Eds.), Lipases, Elsevier. pp. 419-439.
Kamimura ES, Mendieta O, Sato HH, Pastore G, Maugeri F (1999). Production of Lipase from *Geotrichum* sp and Adsorption Studies on Affinity Resin. Braz. J. Chem. Eng. 16(2):103-112.
Kilcawley KN, Wilkinson MG, Fox PF (2002). Determination of key enzyme activities in commercial peptidase and lipase preparations from microbial or animal sources. Enzyme Microb. Technol. 31:310-320.
Klopfenstein WE, Walker HS (1983). Efficiencies of various esters of fatty acids as diesel fuels. J. Am. Oil Chem. Soc. 60:1596-l598.
Kusdiana D, Saka S (2001). Kinetics of transesterification in rapeseed oil to biodiesel fuel as treated in supercritical methanol. Fuel 80:693-698.
Kyndt T, Romeijn-Peeters E, Van Droogenbroeck B, Romero- Motochi JP, Godelieve Gheysen G, Goetghebeur P (2005). Species relationships in the genus Vasconcellea (*Caricaceae*) based on molecular and morphological evidence. Am. J. Bot. 92:1033-1044.
Leal MC, Cammarota MC, Freire DM, Anna Jr S (2002). Hydrolytic Enzymes as Coadjuvants in the Anaerobic Treatment of Dairy Wastewaters. Braz. J. Chem. Eng. 19(2):175-180.
Lee JH, Kim SB, Kang SW, Song YS, Park C, Han SO, Kim SW (2011). Biodiesel production by a mixture of *Candida rugosa* and *Rhizopus oryzae* lipases using a supercritical carbon dioxide process. Bioresour. Technol. 102:2105-2108.
Lee KT, Foglia TA (2000). Synthesis, purification, and characterization of structured lipids produced from chicken fat. J. Am. Oil Chem. Soc. 77:1027-1034.
Lotero E, Liu Y, Lopez DE, Suwannakarn K, Bruce DA, Godwin JG (2005). Synthesis of biodiesel via acid catalysis. Ind. Eng. Chem. Res. 44:5353-63.
Lynn KR, Clevette-Radford NA (1987). Biochemical properties of latices from the Euphorbiaceae. Phytochemistry 26:939-944.
Ma F, Hanna MA (1999). Biodiesel production: A review. Bioresour.

Technol. 70:1-15.
Ma F, Clements LD, Hanna MA (1998). Biodiesel fuel from animal fat: Ancillary studies on transesterification of beef tallow. Ind. Eng. Chem. Res. 37:768-3771.
Mangos TJ, Jones KC, Foglia TA (1999). Lipase-catalyzed synthesis of structured low-calorie triacylglycerols. J. Am. Oil Chem. Soc. 76:1127-1132.
Marchetti JM, Miguel VU, Errazu AF (2007). Possible methods for biodiesel production. Renew. Sustain. Energy Rev. 11:1300-1311.
Mc Carthy P, Rasul MG, Moazzem S. (2011). Analysis and comparison of performance and emissions of an internal combustion engine fuelled with petroleum diesel and different biodiesels. Fuel; 90: 2147–57.
Medecine douce - Medecine africaine (2014). http://www.afriquebio.com/pages/plantes-medicinales-d-afrique/holarrhena-floribunda.html (Consulted on 13/07/2014).
Meher LC, Vidya Sagar D, Naik SN (2006). Technical aspects of biodiesel production by transesterification - A review. Renew. Sustain. Energy Rev. 10:248-268.
Mendes AA, Oliveira PC, de Castro HF (2012). High cell density fed-batch fermentations for lipase production: Feeding strategies and oxygen transfer. J. Mol. Catal. B Enzym. 78:119-134.
Merçon F, Erbes VL, Sant'Anna JGL, Nobrega R (1997). Lipase Immobilized Reactor Applied to Babassu Oil Hydrolysis. Braz. J. Chem. Eng. 14:1.
Ming R, Hou S, Feng Y, Yu Q, Dionne-Laporte A, Saw JH, Senin P, Wang W, Ly BV, Lewis KLT (2008). The draft genome of the transgenic tropical fruit tree papaya (*Carica papaya Linnaeus*). Nature 452:991-996.
Moulin A, Teissere M, Bernard C (1994). Lipases of the euphorbiaceae family purification of a lipase from *Euphorbia characias* latex and structure-function-relationships with the β-chain of ricin. Proc. Natl. Acad. Sci. U.S.A. 91(24):11328-11332.
Mounguengui RWM, Brunschwig C, Baréa B, Villeneuve P, Blin J (2013). Are plant lipases a promising alternative to catalyze transesterification for biodiesel production? Prog. Energy Combust. Sci. 39:441-456.
Mukherjee KD, Hills MJ (1994). Lipases: their structure, biochemistry and application. Biochem. Appl. pp. 49-75.
Mukherjee KD, Kiewitt I (1996). Specificity of *Carica papaya* latex as biocatalyst in the esterification of fatty acids with 1-butanol. J. Agric. Food Chem. 44:1948-52.
Mustranta A, Forsell P, Poutanen K (1995). Comparison of lipases and phospholipases in the hydrolysis of phospholipids. Process. Biochem. 30:393-401.
Ng IS, Tsai SW (2005). Hydrolytic resolution of (R, S)-naproxen 2, 2, 2-trifluoroethyl thioester by *Carica papaya* lipase in water-saturated organic solvents. Biotechnol. Bioeng. 89:88-95.
Ngando Ebongue GF, Dhouib R, Carrière F, Amvam Zollo PH, Arondel V (2006). Assaying lipase activity from oil palm fruit *(Elaeis guineensis Jacq.*) mesocarp. Plant Physiol. Biochem. 44:611-617.
Ogbonnia S, Adekunle AA, Bosa MK, Enwuru VN (2008). Evaluation of acute and subacute toxicity of *Alstonia congensis* Engler (Apocynaceae) bark and *Xylopia aethiopica* (Dunal) A. Rich (Annonaceae) fruits mixtures used in the treatment of diabetes. Afr. J. Biotechnol. 7(6):701-705.
Oliveira JS, Costa-Lotufo LV, Bezerra DP, Alencar NMN, Marinho-Filho JDB, Figueiredo IST, Moraes MO, Pessoa C, Alves APNN, Ramos MV (2010). In vivo growth inhibition of sarcoma 180 by latex proteins from Calotropis procera. 382:139-149.
Olivier M, Zerbo P, Boussim JI, Guinko S (2012). Les plantes des galeries forestières à usage traditionnel par les tradipraticiens de santé et les chasseurs Dozo Sénoufo du Burkina Faso. Int. J. Biol. Chem. Sci. 6(5):2170-2191.
Padiglia A, Medda R, Lorrai A, Murgia B, Pedersen JZ, AgroÂ AF, Floris G (1998). Characterization of *Euphorbia characias* Latex Amine Oxidase. Plant Physiol. 117:1363-1371.
Palocci C, Soro S, Cernia E, Fiorillo F, Belsito C MA, Monacelli B, Monache GD, Pasqua G (2003). Lipolytic isoenzymes from *Euphorbia* latex. Plant Sci. 165:577-582.
Paques FW, Macedo GA (2006). Plant lipases from latex: Properties and industrial applications. Quim. Nova 29:93-99.
Pereira EP, Zanin GM, Castro HF (2003). Immobilization and Catalytic Properties of Lipase on Chitosan for Hydrolysis and Esterification Reactions. Braz. J. Chem. Eng. 20(4):343-355.
Pleiss J, Fischer M, Schmid RD (1998). The Lipase Engineering Database: a navigation and analysis tool for protein families. Chem. Phys. Lipids 93:67-80.
Pradhan A, Shrestha D, McAloon A, Yee W, Haas M, Duffield J (2009). Energy life-cycle assessment of soybean bio-diesel. United States Department of Agri- culture. Agricultural economic report.
Ranganathan SV, Narasimhan SL, Muthukumar K (2008). An overview of enzymatic production of biodiesel. Bioresour. Technol. 99:3975-3981.
Robles-Medina A, Gonzalez-Moreno PA, Esteban-Cerdan L, Molina-Grima E (2009). Biocatalysis: Towards ever greener biodiesel production. Biotechnol. Adv. 27:398-408.
Sadeghinezhad E., Kazi S.N., Badarudin A., Oon C.S., Zubir M.N.M., Mehrali Mohammad (2013). A comprehensive review for biodiesel as alternative fuel for compression ignition engines. Renewable and Sustainable Energy Reviews 28; 410–424.
Saka S, Kusdiana D (2001). Biodiesel fuel from rapeseed oil as prepared in supercritical methanol. Fuel 80:225-231.
Santana GCS, Martins PF, Silva NL, Batistella CB, Filho RM, Maciel MRW (2010). Simulation and cost estimate for biodiesel production using castor oil. Chem. Eng. Res. Des. 88:626-632.
Seth S, Chakravorty D, Dubey VK, Patra S (2014). An insight into plant lipase research – Challenges encountered. Protein Express Purif. 95:13-21.
Shimada Y, Watanabe Y, Samukawa T, Sugihara A, Noda H, Fukuda H, Tominaga Y (1999). Conversion of vegetable oil to biodiesel using immobilized *Candida Antarctica* lipase. J. Am. Oil Chem. Soc. 76:789-93.
Shimada Y, Watanabe Y, Sugihara A, Tominaga Y (2002). Enzymatic alcoholysis for biodiesel fuel production and application of the reaction to oil processing. J. Mol. Catal. B Enzym. 17:133-142.
Simons JW, Egmond MR, Gotz F, Verheij HM (1998). Biochemical properties of staphylococcal (phospho) lipases. Chem. Phys. Lipids 93:27-37
Sokpon N, Lejoly J (1996). Les plantes à fruits comestibles d'une forêt semi-caducifoliée: Pobè, au Sud-est du Bénin. In: L'alimentation en forêt tropicale - Interactions bioculturelles et perspectives de développement. Volume 1: Les ressources alimentaires: Production et consommation. Editions UNESCO MAB. pp. 315-324.
Soumanou MM, Bornscheuer UT (2003). Improvement in lipase-catalyzed synthesis of fatty acid methyl esters from sunflower oil. Enzyme Microb. Technol. 33:97-103.
Soumanou MM, Djenontin ST, Tchobo FP, Sohounhloue DC, & Bornscheuer UT (2012). Lipase-catalysed biodiesel production from Jatropha curcas oil. Lipid Technology, 24(7):158-160.
Sousaa JS, Cavalcanti-Oliveiraa EA, Gomes ADA, Freire DMG (2010). Application of lipase from the physic nut (*Jatropha curcas* L.) to a new hybrid (enzyme/chemical) hydroesterification process for biodiesel production. J. Mol. Catal. B Enzym. 65:133-137.
Srivastava A, Prasad R (2000) Triglycerides-based diesel fuels. Renew. Sustain. Energy Rev. 4:111-133.
Tamboura HH, Bayala B, Lom M, Guissou IP, Sawadogo L (2005). Ecological distribution, morphological characteristics and acute toxicity of aqueous extracts of *Holarrhena floribunda* (g. don) Durand & Schinz, *Leptadenia hastate* (pers.) decne and *Cassia sieberiana* (d c) used by veterinary healers in Burkina Faso. Afr. J. Tradit. CAM. 2(1):13-24.
Tan TW, Lu JK, Nie KL, Deng L, Wang F (2010). Biodiesel production with immobilized lipase: A review. Biotechnol. Adv. 28:628-634.
Tecelão C, Rivera I, Sandoval G, Ferreira-Dias S (2012). *Carica papaya* latex: A low-cost biocatalyst for human milk fat substitutes production. Eur. J. Lipid Sci. Technol. 114:266-276.
Thirstrup K, Verger R, Carrière F (1994). Evidence for a pancreatic lipase subfamily with new kinetic properties. Biochemistry 33:2748-2756.
Turner R (1995). Euphorbias. A gardeners' guide. London: Batsford. pp. 87-93.
Varma MN, Madras G, Chem J (2008). Kinetics of synthesis of butyl butyrate by esterification and transesterification in supercritical

carbon dioxide. Technol. Biotechnol. 83:1135-1144.
Villeneuve P (2003). Plant lipases and their applications in oils and fats modification. Eur. J. Lipid Sci. Technol. 105:308-317.
Villeneuve P, Pina M, Montet D, Graille J (1995). *Carica papaya* latex lipase: sn3 stereospecificity or short chain selectivity? Model chiral triglycerides are removing the ambiguity. J. Am. Oil Chem. Soc. 72:753-755.
Villeneuve P, Pina M, Skarbek A, Graille J, Foglia T (1997a). *Carica papaya* latex-catalyzed synthesis of structured triacylglycerols. Biotechnol. Tech. 11:91-94.
Villeneuve P, Skarbek A, Pina M, Graille J, Foglia TA (1997b). Catalytic behavior of *Carica papaya* latex in transesterification reactions. Biotechnol. Tech. 11:637-639.
Villeneuve P, Turon F, Caro Y, Escoffier R, Barea B, Barouh B, Lagoa R., Piomboa G, Pinaa M (2005). Lipase-catalyzed synthesis of canola phytosterols oleate esters as cholesterol lowering agents. Enzyme Microb. Technol. 37:150-155.
Winayanuwattikun P, Kaewpiboon C, Piriyakananon K, Chulalaksananuku W, Yongvanich T, Svasti J (2011). Immobilized lipase from potential lipolytic microbes for catalyzing biodiesel production using palm oil as feedstock. Afr. J. Biotechnol. 10:1666-1673.
Wright H J, Segur JB, Clark HV, Coburn SK, Langdon EE, Du Puis RN (1944). A report on ester interchange. Oil Soap 21:145-148.
Wu WH, Foglia TA, Marmer WN, Phillips JG (1999). Optimizing production of ethyl esters of grease using 95% ethanol by response surface methodology. J. Am. Oil Chem. Soc. 76(4):571-621.
Xu X (2000). Production of specific-structured triacylglycerols by lipase-catalyzed reactions: A review. Euro. J. Lipid Sci. Technol. 102:287-303.

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Full Length Research Paper

Micropropagation of *Guadua angustifolia* Kunth (Poaceae) using a temporary immersion system RITA®

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The micropropagation of *Guadua angustifolia* Kunth, commonly known as giant bamboo, using semi-solid culture medium, is reported to have low multiplication rates. This study evaluated the multiplication index of *G. angustifolia* in a temporary immersion system (RITA®), comparing results with those obtained using a semi-solid culture medium. The treatments consisted of either three or four 2-min immersions per day and use of semi-solid culture medium, which consisted of MS supplemented with 3.0 mg L^{-1} of the cytokinin benzylaminopurine (BAP). Equipment consisted of 20 vessels for automated RITA®, each containing 200 ml of culture medium. Immersions were performed for 2 min at two different frequency intervals (6 and 8 h). Large clumps of *G. angustifolia* with 1, 2 or 3 stems were inoculated depending on the treatment. Best results were obtained with four immersion cycles per day (every 6 h), with a multiplication index of 2.7 shoots per original explant (axillary buds) and greater rhizome growth. Overall, the temporary immersion system performed better than the semi-solid medium in terms of shoot multiplication rates and rhizome growth. Further studies should be conducted to develop an application for RITA® for use in the commercial production of *G. angustifolia*.

Key words: Giant bamboo, temporary immersion system RITA, rhizome.

INTRODUCTION

Guadua angustifolia, also known as giant bamboo, belongs to the Poaceae family, one of the four largest families of the plant kingdom, harboring from 600 to 700 genera and nearly 10,000 species (Soderstrom et al., 1988). In Colombia, most populations of *G. angustifolia* grow between 0 and 1800 m altitude, occupying diverse habitats of tropical moist forests (Bh-T) and premontane wet forests (Bmh-P), where they form small patches along rivers or streams (Londoño, 1990).

Because of its widespread distribution, versatility and

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physical and mechanical characteristics, *G. angustifolia* has played an important environmental, cultural and economic role in Colombia. However, for these same reasons, it has also suffered indiscriminate exploitation. Enormous pressure has been exerted on natural populations of this species, reducing the areas planted and endangering not only the species but also other associated organisms (Cruz, 2009). Furthermore, *G. angustifolia* produces seeds with low germination capacity, which makes sexual reproduction difficult. The deterioration of stands of *G. angustifolia* during vegetative multiplication is further justification for exploring micropropagation approaches to develop efficient mass multiplication methods for this important species using explants (Cruz, 2009).

Propagation studies are appropriate in the case of *G. angustifolia* because *in vitro* multiplication techniques are an alternative for species conservation, providing abundant high-quality planting material. Manzur (1988) reported that *G. angustifolia* plants perform similarly in *in vitro* and *in situ* conditions. As a result, when micro-cuttings from lateral branches are cultured in enriched culture medium, their axillary buds activate the development of intercalary meristems with roots emerging from their basal nodes. The rhizome is immediately formed, which then becomes the fundamental structure in the micropropagation of *G. angustifolia*.

Only a few studies have been reported on the micropropagation of *G. angustifolia* (Manzur, 1988; Marulanda et al., 2005; Jiménez et al., 2006; Daquinta et al., 2007). Most studies conducted on micropropagation of bamboo have focused on Asian species (Muñoz et al., 1989; Saxena, 1990; Gielis and Oprins, 2000; Sood et al., 2002; García et al., 2007; Lin et al., 2007; Ogita et al., 2008; Venkatachalam et al., 2015; Gantait el al., 2016).

In Colombia, several studies have been conducted on the micropropagation of *G. angustifolia*, achieving partial success at different stages of solid culture medium. The first reports by Manzur (1988) showed low multiplication rates (less than 2 shoots/explant); however, Marulanda et al. (2005) subsequently reported a survival rate of 30% during the establishment phase and Acosta and Guzmán (1993) reported a better multiplication rate when 5.0 mg L^{-1} 6-benzylaminopurine (BAP) was added to the culture medium, supplemented with kinetin 1.0 mg L^{-1} and indoleacetic acid (IAA) 1.0 mg L^{-1}.

Subsequently, Marulanda et al. (2005) and Jiménez et al. (2006) used BAP (2.5 to 5.0 mg L^{-1}) as growth regulator, reporting low multiplication rates. Large losses have also been recorded during the establishment phase of *G. angustifolia* because of bacteria-induced contamination (Cruz et al., 2007; Ramírez et al., 2009).

Depending on the consistency of the culture medium (liquid, semi-solid and solid) used in plant micro-propagation, several systems have been developed to increase the multiplication index and reduce costs. Taking into account the liquid condition of the medium, a system was initially designed that consisted of a large, elevated culture chamber that could be drained and then refilled with fresh medium (Tisserat and Vandercook, 1985). Subsequently a semi-automated system was developed in which plants were cultured in a large container on a medium that contained no gelling agent, with automatic addition and removal of liquid medium at fixed intervals so the plant material entered into contact with the medium several times throughout the day (Aitken-Christie and Davies, 1988).

Simonton et al. (1991) developed a programmable apparatus, which intermittently applied culture medium to the plants according to a pre-defined schedule, serving as basis for the temporary immersion system (TIS) for plant propagation (Alvard et al., 1993). This system, whose commercial name is RITA for recipient for automated TIS, has been successfully used with many plant species, achieving significant increases in multiplication rates and allowing the semi-automation of processes (Lorenzo et al., 1998). Furthermore, the automation of one or more phases of micropropagation can help reduce not only handling costs but also costs related to laboratory space, while increasing production volume (Castro and González, 2000; Capote et al., 2009).

The frequency and time conditions required to achieve efficiency in TIS processes are determinants for system optimization (Etienne and Berthouly, 2002). Albarrán et al. (2002) confirmed that the massive regeneration of somatic embryos of coffee (*Coffea arabica*) improved when exposure in terms of frequency and duration was optimized. Increases in daily frequency (1×1', 2×1', 6×1') stimulated embryo production without affecting embryo quality.

The TIS (Figure 1A and 1B) offers multiple advantages as compared with propagation using semi-solid culture media (Figure 1C) in terms of shoot multiplication in different plant species (González et al., 2005; Roels et al., 2006; Barberini et al., 2011). In the case of banana, increases have been achieved in high average multiplication rates, with improved plant quality (Albany et al., 2005; Berthouly and Etienne, 2005; Watt, 2012; Pérez et al., 2013). Only a few bamboo species such as *Dendrocalamus latiflorus* (Mongkolsook et al., 2005 cited by García-Ramirez et al., 2014), *Bambusa ventricosa* and *Dracaena deremensis* (Chaille, 2011) and *Bambusa vulgaris* (García-Ramírez et al., 2014) have been propagated by TIS. This study aimed to optimize shoot production for micropropagation of *G. angustifolia* for commercial purposes.

MATERIALS AND METHODS

Plant material

The study was carried out at the Plant Biotechnology Laboratory of the Universidad Tecnológica de Pereira (UTP), where test plants

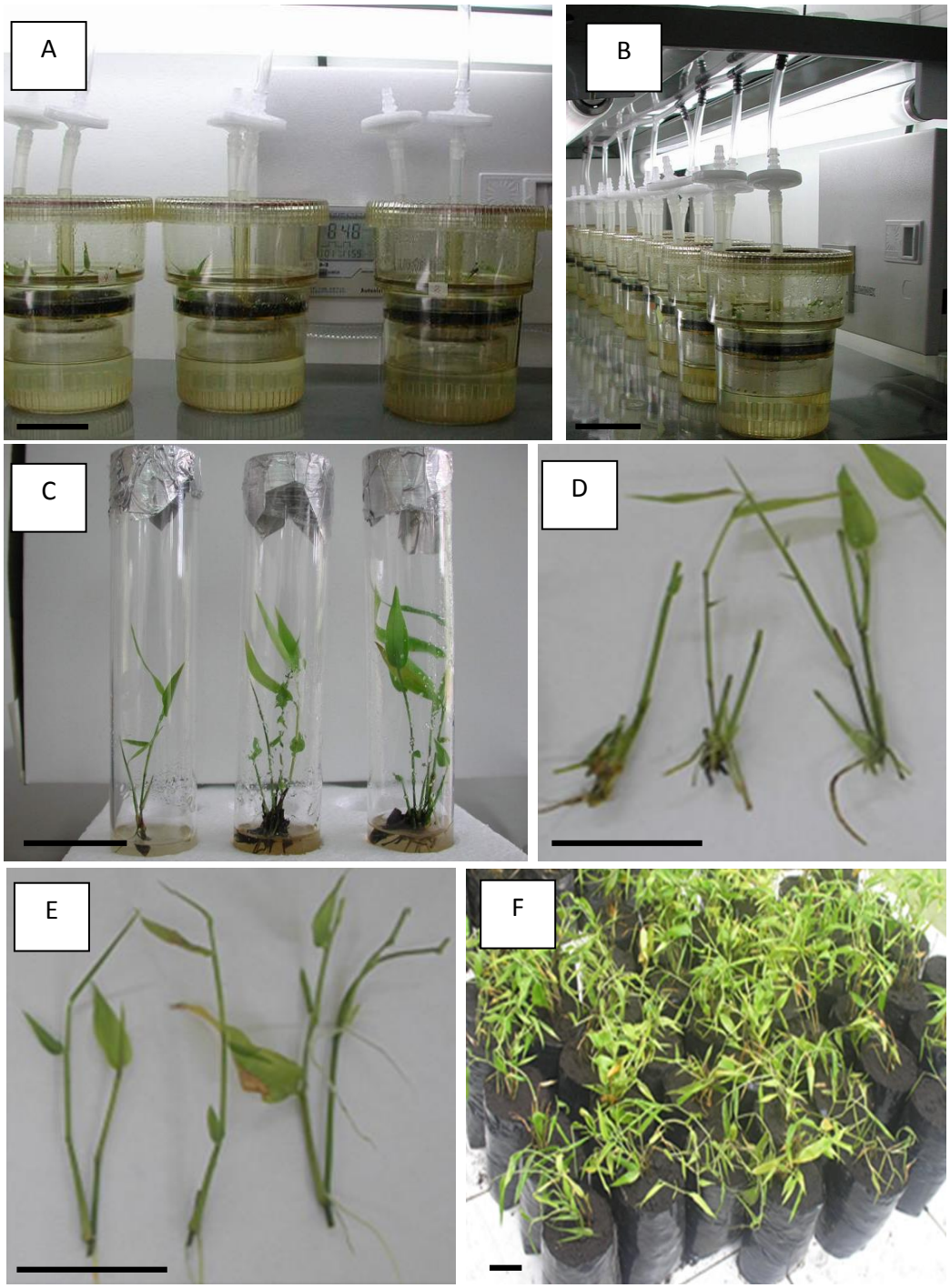

Figure 1. *Guadua angustifolia* shoot multiplication in RITA® (A and B). Shoots of *G. angustifolia* in semi-solid medium (C). Plantlets of *G. angustifolia* obtained from semi-solid medium: non-segmented (presence of rhizome) (D) and segmented (absence of rhizome) (E) and *ex-vitro* acclimatization of plant produced (F). Scale bar, 5 cm.

had been previously established *in vitro*. The study compared the multiplication indexes of *G. angustifolia* plants submitted to TIS with the indexes reached when plants were cultured in semi-solid medium (SSM). Explants, measuring 5 to 10 cm, were inoculated according to the protocol proposed by Marulanda et al. (2005).

Culture conditions

The culture medium, containing MS salts and vitamins, was supplemented with 100 mg L^{-1} myo-inositol and 30 g L^{-1} sucrose. Gelrite® was used as gelling agent in the solid medium at 2.5 g L^{-1},

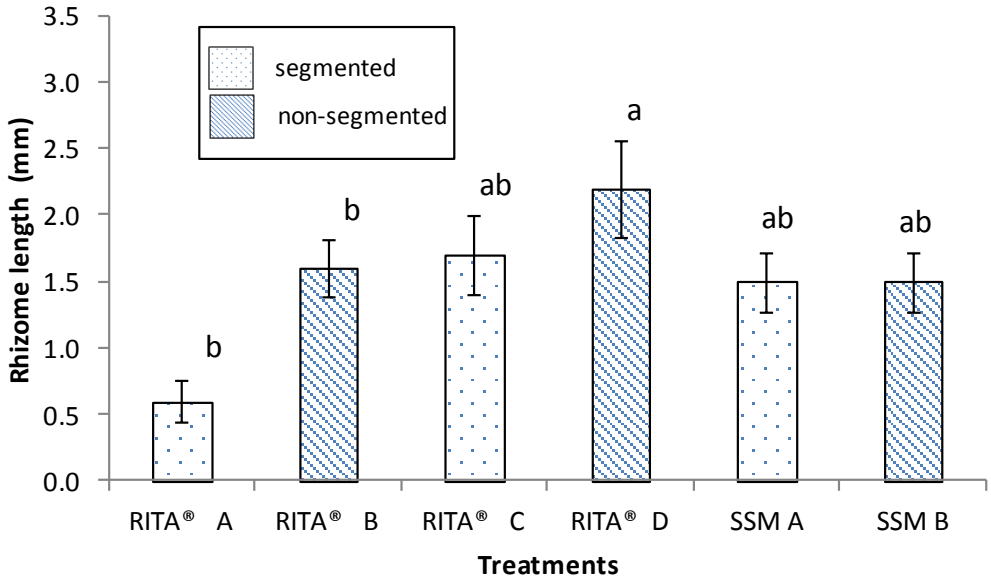

Figure 2. Rhizome size in *Guadua angustifolia* plants depending on culture media and condition of plantlet during four weeks of culture, where RITA® A = 3 cycles, segmented; RITA® B = 3 cycles, non-segmented; RITA® C = 4 cycles, segmented; RITA® D = 4 cycles, non-segmented; SSM A = semi-solid medium, segmented; SSM B = semi-solid medium, non-segmented. Different letters indicate statistically significant differences between groups (mean ± standard error, n = 10, Tukey test, $p<0.05$).

and BAP was used as growth regulator at a concentration of 3 mg L^{-1}.

A total of 200 ml MS basal medium was added to each culture vessel containing liquid medium (TIS) (Figure 1A and 1B), whereas 50 ml were added to each vessel (200 ml) containing SSM. Vessels of both treatments were autoclaved at 121°C at 15 psi, using RITA® vessels developed by CIRAD (Teisson and Alvard, 1995) (Figure 1A and B), being left for 30 min in the case of TIS and 20 min in the case of SSM.

Five explants were inoculated per TIS vessel, whereas only one explant was inoculated per SSM vessel. All plants were placed in a growth chamber with an average temperature of 24±2°C and a 12-h artificial photoperiod (lux).

The multiplication index of *G. angustifolia* plants using the TIS, which consisted of three or four 2-min immersions per day (A = 3 cycles, segmented; B = 3 cycles, non-segmented; C = 4 cycles, segmented; D = 4 cycles, non-segmented), was evaluated and compared with the multiplication index of plants cultured in SSM (A = segmented; B = non-segmented). Large 5 to 10 cm clumps of segmented (absences of rhizomes) and non-segmented (presence of rhizomes) of *G. angustifolia* were used in the inoculation. The number of stems was used as basis to evaluate rhizome generation, shoot multiplication index, and increase in plant height over a 4-week period. Rhizome division was based on the following variables: large clumps, either segmented (Figure 1D) or non-segmented (Figure 1E), and number of stems (1, 2 and 3) per inoculation unit.

Statistical analysis

A completely randomized block design (10 blocks) was used, with each block corresponding to one of the established plants (approximately 40 mm long) and a factorial arrangement of two controlled factors: culture medium and conditions of plants at planting.

Data were analyzed using a random block design with 20 replicates (explants). Each replicate corresponded to one explant and each variable depended on the study factors (rhizome size, shoot multiplication index and increase in plant height after 4 weeks of culture). The data obtained were submitted to the Tukey test to compare the means of the applied treatments.

RESULTS AND DISCUSSION

Effect of culture system on rhizome size for segmented and non-segmented *G. angustifolia*

Analysis of variance indicated that highly significant differences exist in rhizome growth for large clumps of segmented and non-segmented *G. angustifolia*, depending on culture medium consistency (TIS or SSM). The Tukey test, designed to make pairwise comparisons among means, indicated that rhizome growth was greater, but non-significant ($p>0.05$) in plants cultured in the TIS with four 2-min immersions per day as compared with plants cultured in the TIS with three 2-min immersions per day or those cultured in SSM. For RITA®, average rhizome growth was greater in non-segmented large clumps than in segmented large clumps, presenting significant differences ($p<0.05$) when 3 immersion cycles was used (Figure 2).

Manzur (1989) confirmed that when the rhizome originates in *in vitro* conditions, the established *G. angustifolia* plant is capable of generating a large group of successive plants. Furthermore, Londoño (1991) considers bamboo rhizomes to be segmented axes,

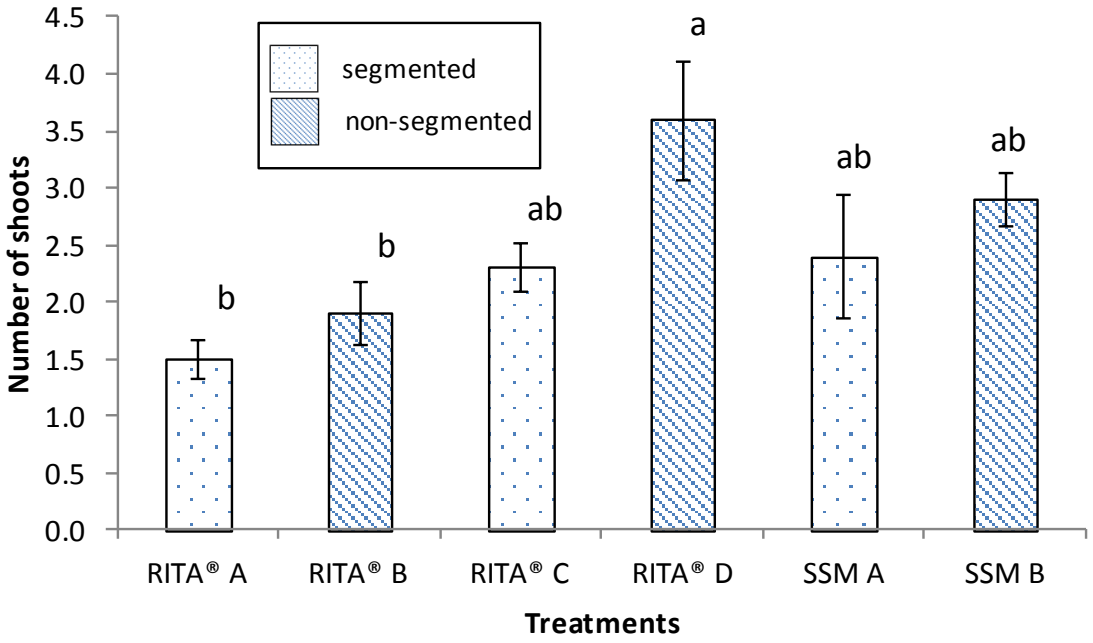

Figure 3. Multiplication of *Guadua angustifolia* shoots depending on culture media and conditions of plantlets during four weeks of culture, where RITA® A = 3 cycles, segmented; RITA® B = 3 cycles, non-segmented; RITA® C= 4 cycles, segmented; RITA® D= 4 cycles, non-segmented; SSM A = semi-solid medium, segmented; SSM B = semi-solid medium , non-segmented. Different letters indicate statistically significant differences between groups (mean ± standard error, n = 10, Tukey test, $p<0.05$).

characterized by having a diameter larger than that of the stem that it will produce. Mostly single rhizome buds appear on nodes from which new plant structures will emerge. Rhizome size was accordingly larger on bamboo plantlets cultured in the TIS with four 2-min immersions per day than when cultured on SSM (Figure 2).

Effect of culture system on shoot multiplication for segmented and non-segmented *G. angustifolia*

The analysis of variance indicated that there are highly significant differences (p=0.0023) in the number of shoots produced per explant depending on culture medium consistency. Significant differences also occurred between segmented and non-segmented explants. For the Tukey test, plantlets cultured in the TIS (Figure 3), with four 2-min immersion cycles, showed a higher, but non-significant (p>0.05) average number of shoots than plants cultured in the TIS with three 2-min immersion cycles and in SSM. Furthermore, non-segmented plants presented a higher number of shoots than segmented plants (Figure 3). These results agree with those of studies carried out in sugarcane (*Saccharum officinarum*) by Lorenzo et al. (1998) and in *Eucalyptus grandis* by Castro and González (2000), two species that presented a higher increase of biomass in the TIS than in solid and semi-solid culture media.

Effect of culture system on growth of segmented and non-segmented *G. angustifolia*

The analysis of variance revealed that the culture medium consistency (TIS or SSM) significantly affected growth of *G. angustifolia*. Highly significant differences in plant height were also observed when segmented and non-segmented plants were compared in RITA® (Figure 4). Plants cultured in SSM presented greater growth than those segmented and cultured in the TIS (four and three 2-min cycles per day). Overall, non-segmented plants presented higher average growth than segmented plants (Figure 4).

Plant elongation during the multiplication phase in the solid culture medium is similar to that reported by Marulanda et al. (2005) and Jiménez et al. (2006). Plant elongation in the TIS (four 2-min immersions per day) is similar to that obtained using the solid culture medium with non-segmented explants.

In contrast, Castro and González (2000) reported that plant size of *E. grandis* increased in the TIS as compared with plants cultured in solid medium. Lorenzo et al. (1998) also found that sugarcane plants grew taller when cultured in TIS (10.29 cm on average) as compared to solid media (6.22 cm on average).

Fifty seedlings cultured in solid media and 50 cultured in TIS were transferred to the nursery using the hardening protocol developed by Marulanda et al. (2005) (Figure

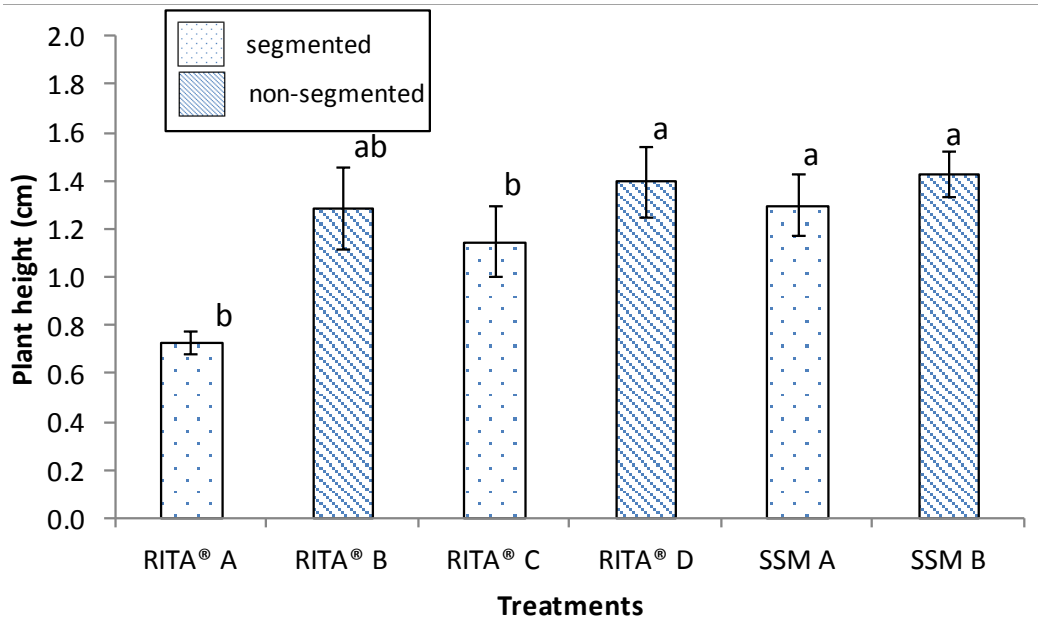

Figure 4. Increase in plant height of *G. angustifolia* plantlets depending on culture media and conditions of plantlets during four weeks of culture, where RITA® A = 3 cycles, segmented; RITA® B = 3 cycles, non-segmented; RITA® C= 4 cycles, segmented; RITA® D= 4 cycles, non-segmented; SSM A = semi-solid medium, segmented; SSM B = semi-solid medium , non-segmented. Different letters indicate statistically significant differences between groups (mean ± standard error, n = 10, Tukey test, $p<0.05$).

1F). No significant differences in plant acclimatization were observed between groups, with a 95% survival rate of plants (data not shown). Plants of *G. angustifolia* from both treatments were then transferred to the field, where plant growth and development are currently being evaluated. Survival rates for other Poaceae in the hardening phase have been reported to be higher than 70% (Gielis and Oprins, 2000; Marulanda et al., 2005; Jiménez et al., 2006).

Effect of culture system on shoot multiplication for segmented and non-segmented *G. angustifolia*

In this study, the average number of shoots produced by plants cultured in the TIS (four 2-min cycles per day) was higher than that of plants cultured in the SSM (Figure 5). The TIS (3 stems) with four 2-min immersions per day produced an average multiplication rate of 3.0 shoots per original explant for non-segmented plants (Figure 5). Marulanda et al. (2005) found an average multiplication rate of 2 shoots per original explant for *G. angustifolia* cultured on solid media, whereas Jiménez et al. (2006) reported an average of 2.5 new individuals (referred to as tillers) per each original plant established. When the averages of all three studies were compared, the higher multiplication efficiency in the TIS reached in this study is clearly demonstrated. In addition, the multiplication rate in solid culture medium yielded results similar to the aforementioned results, with an average multiplication rate of 2.7 shoots per explant.

Using micropropagation in the liquid system with species of the subfamily Bambusoideae, Das and Pal (2005) with *Bambusa balcooa* and Shirin and Rana (2007) with *Bambusa glaucescens* reported successful work in the *in vitro* multiplication phase. In addition, bamboo species such as *Dendrocalamus latiflorus* (Mongkolsook et al., 2005), *Bambusa ventricosa* and *Dracaena deremensis* (Chaille, 2011) and *Bambusa vulgaris* (Garcia-Ramirez, 2014) have been propagated by TIS, with results similar to those found for *G. angustifolia* in terms of rate of multiplication (3 to 5 shoots per explant).

There are similar reports for other species. According to Lorenzo et al. (1998), the *in vitro* multiplication of sugarcane shoots per plant reached higher levels (average of 8.13 shoots) in the TIS than when a solid culture medium was used (average of 3.96 shoots). Yan et al. (2010) also achieved better results with *Siraitia grosvenorii* when RITA® was used for plant regeneration than with solid culture media.

In this study, the number of shoots emerging from the rhizome was higher for both SSM and RITA when plants were cultured with three stems than when the culture was performed with two and one stems per plant. When cultured in the TIS (four cycles per day), *G. angustifolia*

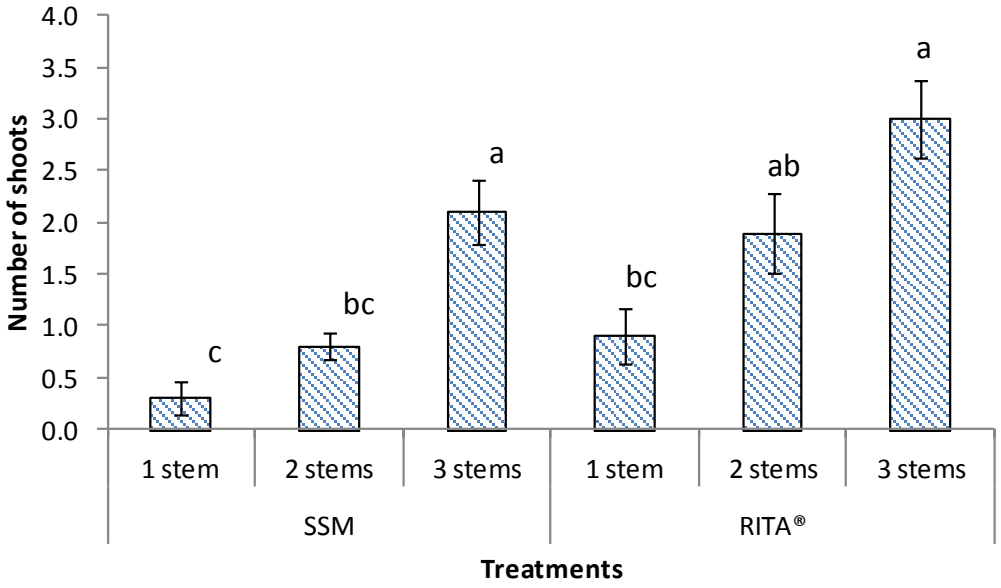

Figure 5. Multiplication rate depending on the number of non-segmented shoots per initial explant inoculated in semi-solid medium and RITA® (four 2-min cycles per day). Different letters indicate statistically significant differences between groups (mean ± standard error, n = 10, Tukey test, $p<0.05$).

plants with three stems produced the highest number of shoots (Figure 5).

For Ravikumar et al. (1998), the number of shoots generated by bamboo plants differs depending on the species and the inoculated explants. Kane (2000) has also found that the type of explants used in micro-propagation processes, whether nodal segments or shoots, stimulate the generation of axillary buds depending on each species.

Conclusion

Multiplying *G. angustifolia* using TIS highlights the importance of the presence of the rhizome (non-segmented explants) for optimal production of shoots. This study also presented good results with a frequency of three 2-min immersions per day, producing 2.7 shoots per original explant. Further studies should be conducted to develop an application for RITA® for use in the commercial production of *G. angustifolia*.

Conflict of interests

The authors have not declared any conflict of interests.

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Abbreviations

BAP, 6-Benzylaminopurine; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **GA,** gibberellic acid; **IAA,** indoleacetic acid; **MS,** Murashige and Skoog (1962); **NAA,** naphthalene acetic acid.

REFERENCES

Acosta V, Guzmán CC (1993). Propagación Vegetativa de la Guadua (*Guadua angustifolia* K.et B. forma “macana”) mediante Cultivo de Tejidos. Trabajo de Grado, Ingeniería Forestal, Universidad del Tolima, Colombia.

Aitken-Christie J, Davies HE (1988). Development of a semi-automated propagation system. Acta Hortic. 230:81-87.

Albany N, Jiménez E, Vilchez J, García L, De Feria M, Pérez N, Sarría Z, Pérez B, Clavelo J (2005). Use of growth retardants for banana (Musa AAA cv. Grand Naine) shoot multiplication in temporary immersion systems. In. Hvoslef AK, Preil W (eds) Liquid Culture Systems for *In Vitro* Plant Propagation, Springer, Dordrecht, Netherlands, pp. 213-224.

Albarrán J, Bertrand B, Vásquez N, Etienne H (2002). La frecuencia y duración de la inmersión afecta la cantidad y calidad de los embriones somáticos de café regenerados en un biorreactor de inmersión temporal. Instituto Nacional de Investigaciones Agrícolas, Centro Nacional de Investigaciones Agropecuarias, Maracay, Venezuela.

Alvard D, Cote F, Teisson C (1993). Comparison of methods of liquid medium culture for banana micropropagation. Plant Cell Tissue Organ Cult. 32:55-60.

Barberini S, Savona M, Ruffoni B (2011). Temporary immersion culture of *Lilium bulbiferum*. Acta Hortic. 900:377-383.

Berthouly M, Etienne H (2005). Temporary immersion system: A new concept for use liquid medium in mass propagation; In: Hvoslef AK, Preil W (eds) Liquid Culture Systems for *In Vitro* Plant Propagation, Springer, Dordrecht, Netherlands, pp. 165-195.

Capote I, Escalona M, Daquinta M, Pina D, González J, Aragon C (2009). Propagación *in vitro* de *Vriesea* en sistemas de inmersión temporal. Biotecnol. Veg. 9:161-167.

Castro D, González J (2000). Eucalyptus (*Eucalyptus grandis* Hill) micropropagación de eucalilto (*Eucalyptus grandis* Hill ex Maiden) en el sistema de inmersión temporal. Agric. Tec. 62:68-78.

Chaille L (2011). Optimization of Tissue Culture Protocols for Cost-Effective Production of Dracaena, Bamboo, and Succulent Plants. M.Sc. Thesis, Department of Tropical Plant and Soil Science, Hawaii.

Cruz H (2009). *Guadua angustifolia* Kunth. Bosques Naturales de Colombia. Plantaciones Comerciales en México. Editorial Gráficas Olímpica S.A., Pereira. Colombia.

Cruz M, García Y, Sánchez C, Alvarado Y, Acosta M, Roque B, Leiva M, Freire M (2007). Identificación y control de *Bacillus* sp., contaminante del establecimiento *in vitro* de *Guadua angustifolia* Kunth. Biotecnol. Veg. 7:9-13.

Daquinta M, Gregori A, Cid M, Lezcano Y, Sagarra F (2007). Formación de callos e inducción de brotes a partir de tejido intercalar de ramas de plantas adultas de *Guadua angustifolia* Kunth. Biotecnol. Veg. 7:119-122.

Das M, Pal A (2005). *In vitro* regeneration of *Bambusa balcooa* Roxb.: Factors affecting changes of morphogenetic competence in the axillary buds. Plant Cell Tissue Organ Cult. 81:109-112.

Etienne H, Berthouly M (2002). Temporary immersion systems in plant micropropagation. Plant Cell Tissue Organ Cult. 69:215-231.

Gantait S, Pramanik B, Banerjee M (2016). Optimization on planting materials for large scale plantation of *Bambusa balcooa* Roxb.: Influence of propagation methods. J. Saudi Soc. Agric. Sci. In Press.

García Y, Freire M, Fajardo L, Tejada M, Reyes M (2007). Establecimiento *in vitro* de yemas axilares de *Bambusa vulgaris* var. Vittata. Biotecnol. Veg. 7:155-159.

García-Ramírez Y, Gonzáles MG, Mendoza EQ, Freire-Seijo M, Cárdenas MLO, Moreno-Bermúdez LJ, Ribalta OH (2014). Effect of BA treatments on morphology and physiology of proliferated shoots of *Bambusa vulgaris* Schrad. Ex Wendl in temporary immersion. Am. J. Plant Sci. 5:205-211.

Gielis, J, Oprins J (2000). Micropropagation of temperate and tropical woody bamboos from biotechnological dream to commercial reality. In: Bamboo for sustainable development. Proceedings of the 5th International Bamboo Congress and the 6th International Bamboo Workshop, San José, Costa Rica, Pp. 333-344.

González JL, Fundora Z, Molina LA, Abdulnour J, Desjardins Y, Escalona A (2005). New contributions to propagation of pineapple (*Ananas comosus* L. Merr) in temporary immersion bioreactors. *In vitro* Cell Dev. Biol. Plant 41:87-90.

Jiménez V, Castillo J, Tavares E, Guevara E, Montiel M (2006). *In vitro* propagation of the neotropical giant bamboo, *Guadua angustifolia* Kunth, through axillary shoot proliferation. Plant Cell Tissue Organ Cult. 86:389-395.

Kane M (2000). Propagation from preexisting meristems. In: Trigano R, Gray D (eds) Plant Tissue Culture Concepts and Laboratory Exercises, 2nd ed. CRC Press, Boca Raton, USA, Pp. 75-97.

Lin ChS, Liang CJ, Hsaio HW, Lin MJ, Chang WC (2007). *In vitro* flowering of green and albino *Dendrocalamus latiflorus*. New For. 34:177-186.

Londoño X (1990). Aspectos sobre la distribución y la ecología de los bambúes de Colombia (Poaceae: bambusoideae). Caldasia 16:139-153.

Londoño X (1991). Estudio botánico, ecológico, silvicultural y económico-industrial de las Bambusoideae de Colombia. Cespedesia 16/17:51-78.

Lorenzo JC, González BL, Escalona M, Teisson C, Espinosa P, Borroto C (1998). Sugarcane shoot formation in an improved temporary immersion system. Plant Cell Tissue Organ Cult. 54:197-200.

Manzur D (1988). Propagación vegetativa de *Guadua angustifolia* Kunth. Rev. Agron. 2:14-19.

Manzur D (1989). Bosques de guadua (Bambusa) en el laboratorio. Rev. Agric. Trop. (Bogotá), pp. 53-55.

Marulanda MM, Gutierrez LG, Márquez M (2005). Micropropagación de *Guadua angustifolia* Kunth. Actual. Biol. 27:5-15.

Mongkolsook Y, Tanasombut M, Sumkaew R, Likitthammanit P, Wongwean P (2005). Temporary Immersion System (TIS) for Micropropagation of *Dendrocalamus latiflorus* in Commercial Production. Kasetsart Agricultural and Agro-Industrial Product Improvement Institute, Kasetsart University, Bangkok.

Muñoz M, Guevara E, Montiel M (1989). Regeneración *in vitro* del bambú gigante *Dendrocalamus giganteus* (Poaceae). Rev. Biol. Trop. 4:50-56.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15:473-479.

Ogita S, Kashiwagi H, Kato Y (2008). *In-vitro* node culture of seedlings in bamboo plant, *Phyllostachys meyeri* McClure. Plant Biotechnol. 25:381-385.

Pérez, MB, Vega VM, Delgado MT, Torres JL, Pino AS, Cabrera AR,Toledo MB, García YB, Ortiz AO (2013). Nueva alternativa para la micropropagación en inmersión temporal del cultivar de plátano vianda "INIVITPV -2011" (AAB) /New alternative for micropropagation in temporary immersion system of plantain cultivar "INIVITPV -2011 (AAB). Rev. Colomb. Biotecnol. 15(1):98-107.

Ramírez LA, Castaño SM, López R (2009). Identificación de bacterias que afectan el establecimiento *in vitro* de segmentos nodales de *Guadua angustifolia* Kunth. Rev. Investig. Univ. Quindio 19:151-158.

Ravikumar R, Ananthakrishnan G, Kathiravan K, Ganapathi A (1998). *In vitro* shoot propagation of *Dendrocalamus strictus* nees. Plant Cell Tissue Organ Cult. 52:189-192.

Roels S, Nocedal C, Escalona M, Sandoval J, Canal MJ, Rodríguez R, Debergh P (2006). The effect of headspace renewal in a temporary immersion bioreactor on plantain (*Musa* AAB) shoot proliferation and quality. Plant Cell Tissue Organ Cult. 84:155-163.

Saxena S (1990). *In vitro* propagation of bamboo (*Bambusa tulda* Roxb.) through shoot proliferation. Plant Cell Rep. 9:431-434.

Shirin F, Rana PK (2007). *In vitro* plantlet regeneration from nodal explants of field-grown culms in *Bambusa glaucescens* Willd. Plant Biotechnol. Rep. 1:141-147.

Simonton W, Robacker C, Krueger S (1991). A programmable micropropagation apparatus using cycled liquid medium. Plant Cell Tissue Organ Cult. 27:211-218.

Soderstrom TR, Judziewicz EJ, Clarck LG (1988). Distribution patterns of neotropical bamboos. In: Heyer WR, Vanzolini PE (eds) Proceedings of the Neotropical Biotic Distribution Pattern Workshop, Academia Brasileira de Ciencias, Rio de Janeiro, Pp. 121-157.

Sood A, Ahuja PS, Sharma M, Sharma OP, Godbole S (2002). *In vitro* protocols and field performance of elites of an important bamboo *Dendrocalamus hamiltonii* Nees et Arn. Ex Munro. Plant Cell Tissue Organ Cult. 71:55-63.

Teisson C, Alvard D (1995). A new concept of plant *in vitro* cultivation liquid medium: Temporary immersion. In: Terzi M, Celia R, Falavigna A (eds) Current Issues in Plant Molecular and Cellular Biology, Kluwer, Dordrecht, Pp. 105-110.

Tisserat B, Vandercook CE (1985). Development of an automated plant culture system. Plant Cell Tissue Organ Cult. 5:107-117.

Venkatachalam A, Kalaiarasi K, Sreeramanan S (2015). Influence of plant growth regulators (PGRs) and various additives on in vitro plant propagation of *Bambusa arundinacea* (Retz.) Wild: A recalcitrant bamboo species. J. Genet. Eng. Biotechnol. 13(2):193-200.

Watt MP (2012). The status of temporary immersion system (TIS) technology for plant micropropagation. Afr. J. Biotechnol. 11:14025-14035.

Yan H, Liang Ch, Li Y (2010). Improved growth and quality of *Siraitia grosvenorii* plantlets using a temporary immersion system. Plant Cell Tissue Organ Cult. 103:131-135.

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Characterization of α-amylase produced by the endophytic strain of *Penicillium digitatum* in solid state fermentation (SSF) and submerged fermentation (SMF)

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α-Amylases are enzymes responsible for breaking the α-1,4 bond in polysaccharides with three or more glucose units, occupying the second place in the most widely employed enzymes in industry in the world. The objective of this study was to compare the yields of α-amylase produced by the endophytic fungus, *Penicillium digitatum*, strain D1-FB, isolated from *Baccharis dracunculifolia* D.C. (Asteraceae), through the solid state fermentation (SSM) and submerged fermentation (SmF) processes, in addition to characterizing the produced enzyme. The two fermentations were conducted for 120 h, taking samples every 24 h to obtain the peaks of production. The enzymes were characterized according to their optimal pH and temperature for performance and stability regarding the incubation in the presence of ions, variations in pH and temperature. The maximum yield of the enzyme was observed with SSF, using rice bran as substrate after 72 h of fermentation, with 1,625 U/mL. The α-amylase had an optimal pH at 6.5 and optimal temperature at 37°C. All the ions resulted in a decrease in the activity of α-amylase in the concentration of 5 mM. The enzyme proved to be quite stable in a pH range of 6.0 to 7.5 and up to the temperature of 37°C, but it presented great drops in activity with temperatures above 45°C and in the presence of ions at the concentration of 5 mM.

Key words: *Penicillium digitatum*, α-amylase, starch, enzymes, endophytic.

INTRODUCTION

Starch is a polymer consisting of glucose molecules joined by α-1,4 and α-1,6 bonds. Two polysaccharides comprise the structure of starch: amylose and amylopectin (Figures 1A and B). The first (1A) is a linear molecule containing more than 6000 glucose units connected by glycosidic α-1,4 bonds, and the second (1B), very similar to glycogen (Myers et al., 2000), is highly branched, containing α-1,4 bonds between the of glucose monomers, and α-1,6 in the branching points at each 24-30 glucose residue (Brena et al., 1996).

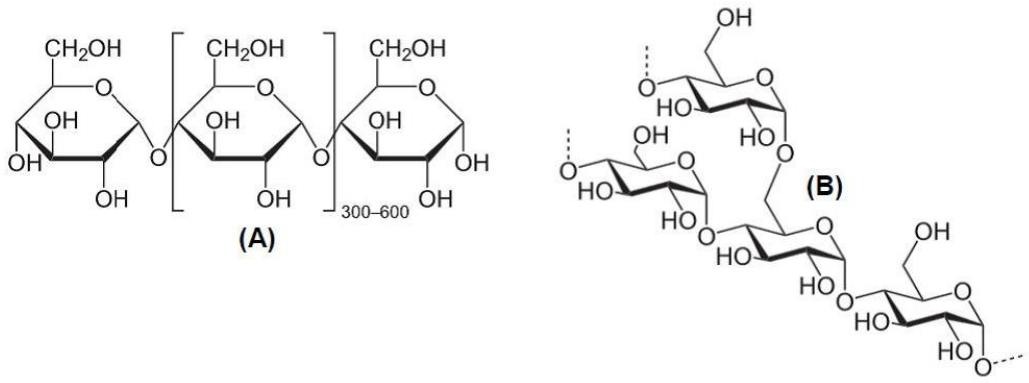

Figure 1. (A) Amylose Molecule. (B) Amylopectin Molecule. Corradini et al. (2005).

Amylases are responsible for the hydrolysis of the starch molecule and are widely distributed in nature. Starch is found mainly in seeds of cereals, such as corn, barley, wheat and rice, and in tubers or roots, such as potatoes and manioc, with the size and shape of the grains being specific for the different cereals (Moraes, 2004).

According to Gupta et al. (2003), amylases are divided into two groups, the endo-amylases and exo-amylases. Endo-amylases catalyze hydrolyses within the starch molecule in various points of the chain simultaneously. This action causes the formation of dextrins and small polymers composed of glucose units of various lengths, breaking the glycosidic α-1,4 bonds present on the inside of the amylose or amylopectin chains. α-Amylase is the most well-known endo-amylase. The exo-amylases known as amiloglicosidases or glicoamylases, hydrolyze glycosidic α-1,4 and α-1,6 bonds (Lorentz, 2005; Onofre et al., 2011, 2012).

Amylases are applied in the most varied industrial sectors that require the hydrolysis of starch, being mainly used in the food industry for the preparation of beers, jellies and to obtain free glucose for the most varied applications (Michelin, 2005).

In addition to the food industry, these enzymes can be used in the formulation of detergents and in the paper, pharmaceutical, fermentation and textiles industries (Oliveira et al., 2007). There are two processes for the production of microbial enzymes: solid state fermentation (FES) and submerged fermentation (SmF). SmF is traditionally used for the production of enzymes because it provides a better control of some important process parameters, such as pH and cell growth, in addition to facilitating the recovery of extracellular enzymes and the determination of biomass (Fernandes, 2006).

One of the main characteristics of solid-state fermentation is the use of substrates with low water activity, in which the conditions for growth are similar to the fungi's natural habitat. This facilitates their growth on the solid substrate and the production of large quantities of enzymes (Paris et al., 2008; Rocha, 2010).

The vast majority of microorganisms used in the solid-state fermentation are filamentous fungi (Silva, 2002). The reduced amount of water in the substrate greatly restricts the number of micro-organisms that are capable of adapting to this process, but fungi prove to be quite tolerant to this environment (Pandey et al., 2005; Fernandes, 2006). The objective of this study was to compare the production of α-amylase produced by the endophytic fungus, *Penicillium digitatum*, strain D1-FB, isolated from *Baccharis dracunculifolia* D.C. (Asteraceae), through the solid state fermentation (SSF) and submerged fermentation (SmF) processes, in addition to characterizing the produced enzyme.

MATERIALS AND METHODS

Micro-organism studied

For the realization of this work, the endophytic fungus *P. digitatum* strain D1-FB was used, isolated from *B. dracunculifolia* D.C. (Asteraceae) maintained in the mycology collection of the Microbiology Laboratory of the Regional Community University of Chapecó – UNOCHAPECÓ – Chapecó – Santa Catarina - Brazil.

Fermentation process for the production of α-amylases

Preparation of the inoculum

After growing the fungus on PDA for seven days, the cells were suspended in a phosphate 100 mM buffer, pH 7.0, and subjected to stirring in order to obtain a homogeneous solution. The cell concentration was determined by counting in a Neubauer chamber (Germano, 2000).

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Preparation of the fermentation media

Two different fermentation processes for the production of amylase were tested: SSF and SmF. The substrate of choice for SSF was rice bran. Each Erlenmeyer flask of 125 mL received 10 g of rice bran (Fa), with the production of the enzyme in pure bran (Fa) being compared with bran supplemented with 20% of manioc starch (Fm).

The moisture content of the media was guaranteed by a phosphate buffer 100 mM, pH 6.5, in the proportion of 65% for the medium containing pure bran, and of around 90% for the medium supplemented with manioc starch. In SmF, the medium was composed of 50 $g.L^{-1}$ of manioc starch, 0.2 $g.L^{-1}$ of $MgSO_4$ and 0.2 $g.L^{-1}$ of $ZnSO_4.7H_2O$, solubilized in a phosphate buffer 100 mM, pH 6.5. Two different sources of nitrogen were tested, one organic (urea) and the other inorganic ($NaNO_2$) at 2.5 $g.L^{-1}$.

The fermentation was conducted in Erlenmeyer flasks of 125 mL containing the medium at 20% of its maximum capacity. 10^8 spores were inoculated in all media. The flasks were incubated in a Shaker at 28°C and 150 rpm. Samples were taken every 24 h for four days for the evaluation of enzyme yields (Onofre et al., 2011; Onofre et al., 2012).

Recovery of the enzyme from the fermented media

For the recovery of the enzyme from the solid fermented medium, 5 mL of NaCl 1% solution was added for each gram of solid substrate and maintained under stirring for 1 h at 100 rpm. For the liquid medium, a solution of NaCl 1% was added until the volume reached 50 mL. After this process, the suspension was filtered to obtain the crude extract, and then centrifuged at 2000 rpm for 8 min, discarding the precipitate.

Enzymatic assay

The activity of α-amylase was determined by measuring the levels of reducing sugars in solution as a result of the action of the α-amylase on the starch. The activity was determined in samples in triplicate by quantifying the reducing sugars (glucose) with the Miller (1959) and Fernandes et al. (2007) method. A mixture containing 0.5 mL of enzymatic extract; 0.5 mL of a starch 0.5% solution in a Tris-HCl buffer 0.05M pH 8.5, and 0.2 mL of the same buffer was incubated at 90°C for 10 min. After this period, 1.0 mL of the Miller reagent (3, 5-dinitrosalicylic acid) was added to the reaction. The mixture was placed in boiling water for 10 min, then cooled in an ice bath for 5 min and 4.8 mL of distilled water was added. The developed color was measured with a SHIMADZU UV-mini 1240 spectrophotometer, using a wavelength of 540 nm. The same procedure was performed with the control, except that the miller reagent (3, 5-dinitrosalicylic acid) was added together with the enzyme to the starch 0.5% solution, and this mixture was placed in boiling water as described above. The content of reducing sugars was determined through a glucose curve. One unit of enzyme activity (U) was defined as the amount of enzyme required to produce 1 µmol of reducing sugar per minute per mL from the soluble starch under the test conditions (Onofre et al., 2011; Onofre et al., 2012).

Characterization of the α-amylase

Optimal pH for enzymatic activity

For the assessment of the influence of pH on enzyme activity, the enzymatic assay was conducted according to item 2.3, while varying the addition of buffer solution: pH 5.0 - 6.0 (acetate buffer), 6.5 - 7.5 (phosphate buffer), 8.0 - 8.5 (Tris-HCl buffer) (Thys, 2004).

Optimal temperature for enzymatic activity

For the assessment of the influence of temperature on enzyme activity, the enzymatic assay was conducted according to item 2.3, while only varying the incubation temperature: 10, 20, 30, 37, 45 and 65°C (Silva, 2002).

Influence of ions on enzymatic activity

For the assessment of the influence of ions on enzyme activity the enzyme was incubated for 10 min at room temperature with the ions $CaCl_2$, $BaCl_2$, $HgCl_2$, $FeCl_3$ and $MgCl_2$ at concentrations of 1 and 5 mM, performing the enzymatic assay after this procedure, as described by Giongo (2006).

Thermal stability of α-amylase

The thermal stability of the amylase was tested by incubating the enzyme for 30 min at the temperatures of: 10, 20, 30, 37, 45 and 65°C, performing the enzymatic assay after this procedure as described by Rasiah and Rehm (2009).

Stability of α-amylase at different pH values

The stability of *α*-amylase at different pH values was tested by incubating the enzyme for 30 min in the following buffer solutions (100 mM): pH 5.0 - 6.0 (acetate buffer), 6.5 - 7.5 (phosphate buffer), 8.0 - 8.5 (Tris-HCl buffer), performing the enzymatic assay after this procedure as described by Thys (2004).

RESULTS AND DISCUSSION

Through the analysis of the fermentation processes, one can observe that the endophytic strain of *P. digitatum*, isolated from *B. dracunculifolia* D.C. (Asteraceae) had better results in the solid medium when compared with fermentation in the liquid medium, yielding 1625 U/mL through solid state fermentation in the medium containing only rice bran, while in submerged fermentation these values did not exceed 712 U/mL in the medium containing inorganic nitrogen (Liq/Ino). By comparing the data obtained in this study with those found by Spier (2005) working with *Penicillium* sp., one can see that he obtained similar results as those of this study, since he observed a production of 1690 U/mL of fungal amylases in SSF, with yields surpassing double the activity achieved in SmF. This same behavior was observed by Hu et al. (2013), who found that the fungus *Penicillium* sp. in a semi-solid medium had better results than in submerged fermentation and in a medium supplemented with an inorganic fraction ($NaNO_2$) of nitrogen.

In Figure 2, the enzyme yield data (in total units) in the different media over the course of 120 h at 24 h intervals is presented.

SSF has been described as an excellent option for the growth of the filamentous fungi. In general, fungi have a

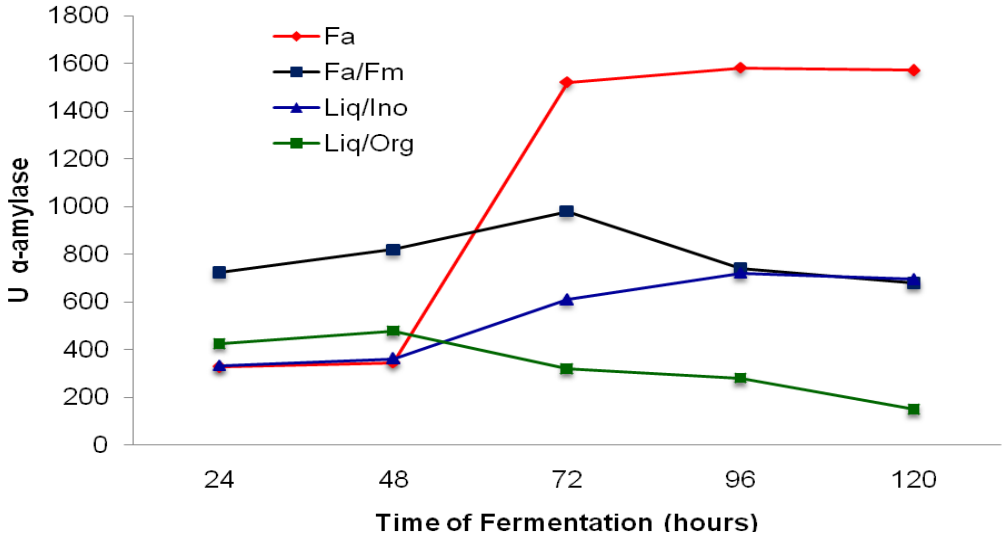

Figure 2. Yield of *α*-amylase as a function of time in solid media containing only rice bran (Fa) and supplemented with manioc starch (Fa/Fm), and in liquid medium with the organic nitrogen source urea (Liq/Org) and the inorganic source $NaNO_2$ (On/Innovation).

good ability to grow on solid substrates, which explains their good adaptation to SSF, and for this reason, they have been widely used in this process, mainly to obtain enzymes (Silva, 2002; Lei et al., 2014).

One of the main characteristics of SSF is the use of substrates with low water activity, in which the conditions for growth are similar to the natural habitat of fungi, which facilitates their growth on the solid substrate and the yield of large quantities of enzymes. The organic matter present in this material is used as a source of energy for growth, and the carbon is used for the synthesis of cellular biomass and of the products of the microbial metabolism (Mitchell and Lonsane, 1992; Hattori et al., 2013; Driss et al., 2014).

It should be noted, however, that the SSF technology should not be seen as a technique that replaces submerged fermentation. In fact, each technique has its own potential and particularities. However, there is a consensus about the need for ongoing research on the factors related to SSF in order to take advantage of the full potential of this technology (Pandey et al., 2001). According to Del Bianchi et al. (2001) and Pandey (2002), the control of certain variables is necessary to obtain products with constant and uniform characteristics. One can therefore state that the observation of these factors, and the correct handling in relation to each one of them, will certainty bring about better results in the solid state fermentation process. Environmental conditions, such as temperature, pH, water activity, oxygen level and the concentration of nutrients and products, significantly affect cell growth and product formation.

When the liquid fermentation media are compared, the fungus can be observed to have adapted better to the inorganic source of nitrogen, showing a maximum yield of 712 U/mL, while in the medium containing organic nitrogen the peak yield of total amylases was 438 U/mL. According to Gupta et al. (2003), organic nitrogen sources are preferred for the production of α-amylase by bacteria. On the other hand, various inorganic salts, such as ammonium sulfate, sodium nitrate and ammonium nitrate, have been reported in improved yields of α-amylase by fungi. Both the solid media had good enzyme yields: 1625 U/mL in the medium containing rice bran, and 932 U/mL in the medium containing rice bran supplemented with manioc starch.

One would expect that the medium supplemented by starch would have higher enzyme yields, since the presence of starch should induce the production of amylolytic enzymes, but this was not the behavior observed. This fact may have occurred due to the presence of starch, which gelatinized after heat treatment (sterilization), making the medium more compact. As such, this made aeration of the solid medium harder. Spier (2005) reported this same limitation with the use of starch in submerged cultivation, noting that enzyme activity decreased with the increase of the concentration of starch in the medium.

Characterization of the enzyme

Optimal pH for enzymatic activity

The *α*--amylase produced by SSF in the medium containing rice bran were used in the tests for characterization of the enzyme. Figures 3 and 4 show the activity of the enzyme as a function of pH and temperature, respectively. The enzyme showed optimal

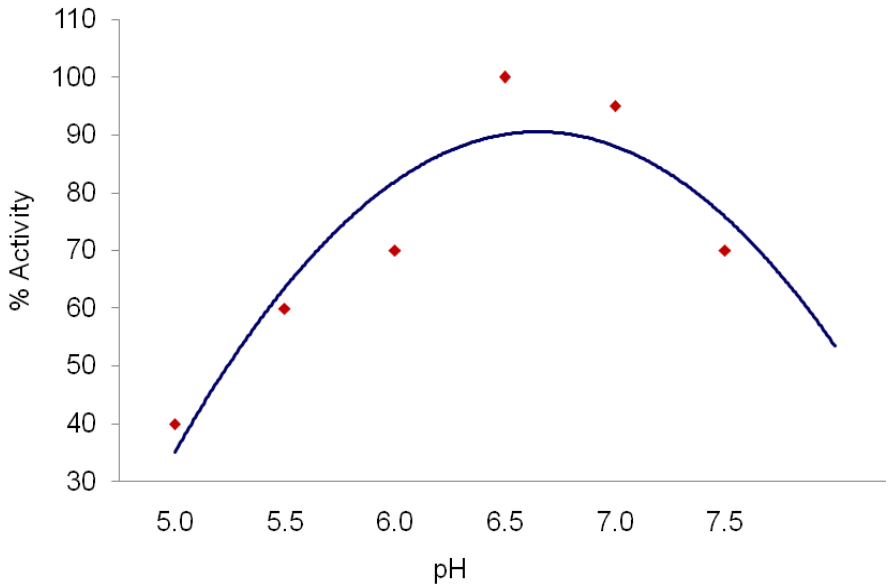

Figure 3. Curve representing the optimal pH for *α*-amylase activity.

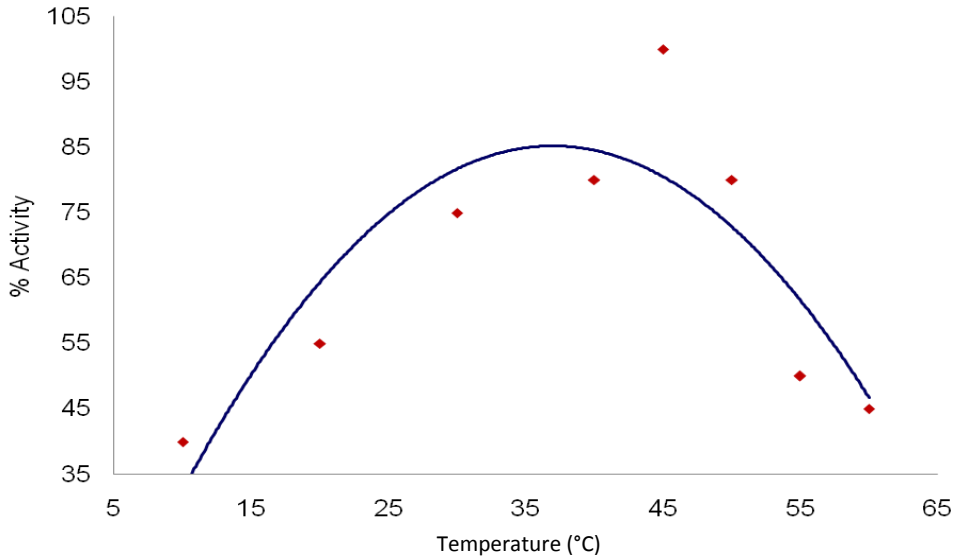

Figure 4. Curve representing the optimal temperature for *α*-amylase activity.

pH at 6.5, while maintaining 96% of its activity at pH 7.0, characterizing it as a neutral *α*-amylase. This data is consistent with Figueira et al. (2000) and Oliveira et al. (2010) who produced amylases with the fungi *Fusarium moniliforme* and *Aspergillus flavus,* obtaining optimal pH values close to 6.9, demonstrating the trend for the production of slightly neutral amylases by filamentous fungi. Spier (2005) on the other hand, obtained a higher yield of amylolytic enzymes with an initial pH equal to 4.0 when working with *Aspergillus niger*, which shows that each species of fungus may have a different behavior in specific pH.

According to Soccol (1992) and Onofre et al. (2011, 2012), the growth capacity of fungi is limited in extreme conditions of acidity and alkalinity. This characteristic is of extreme importance in fermentation processes since they show that under these conditions the vast majority of the bacteria responsible for the contamination of fermentation processes are inhibited. The optimal temperature was 37°C, with the enzyme being completely inactivated at the temperature of 65°C. Spier (2005) obtained similar results to those in this study for a fungal amylase,

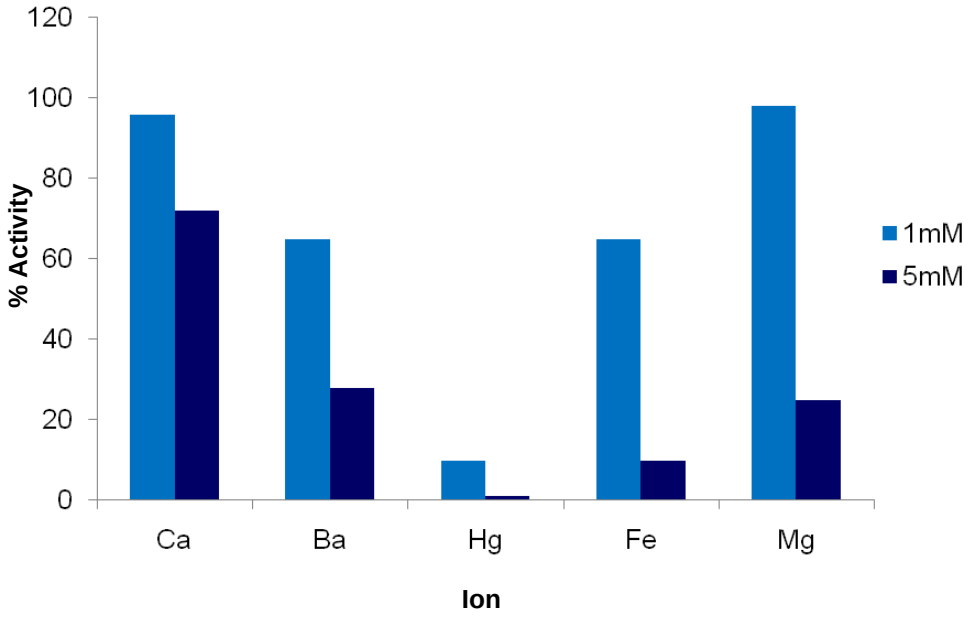

Figure 5. Influence of ions on enzyme activity at the concentrations of 1 and 5 mM.

reporting optimal pH at 6.0 and optimal temperature between 45-55°C. This behavior is also in agreement with Kundu et al. (1973) and Ueno et al. (1987) cited by Pandey et al. (2005), who reported that optimal yields of α-amylase were obtained at temperatures between 30 and 37°C.

Influence of Ions on enzymatic activity

Metal ions have a variety of functions in proteins. These ions may be directly involved in the catalytic process during the enzymatic reaction, or they may participate in electron transfer or redox reactions (Najafi et al., 2005). The stabilization of some enzymes can be induced, mainly, by such divalent ions as Ca^{2+}, Mn^{2+}, Zn^{2+} and Mg^{2+}. If used in low concentrations, these ions can stabilize the tertiary structure of the protein, promoting the formation of cross-links that provide a greater stability to it (Tomazic, 1991). Figure 5 shows the behavior of *α*-amylase regarding the incubation with some ions. All the ions tested at concentrations of 1 and 5 mM were associated with a drop in activity of the enzyme of at least 14%, with the ions Hg^{2+}, at both concentrations, and Fe^{3+}, at the concentration of 5 mM, leading to the complete loss of enzyme activity. The change in activity per ion is a very particular characteristic of each enzyme. Some enzymes may require divalent ions for their activation or as a cofactor, commonly Ca^{2+}, Mg^{2+}, Zn^{2+} and Mn^{2+}, increasing their activity. However, some ions may generate a drastic drop in activity, such as Hg^{2+}, Fe^{3+}, and may lead to total inhibition (Giongo, 2006). It should be noted that high concentrations of ions can have an inhibitory effect. Some studies also show that the stability of α--amylase is compromised in the presence of small quantities of these ions (Yang and Liu, 2004; Bernhardsdotter et al., 2005; Hashim et al., 2005).

Thermal stability of α-amylase and at different pH values

pH is one of the most important factors that affect fermentation processes, since it can change the chemicals of the culture medium, ionize polar molecules, affect enzymatic reactions and the post-translation processes of enzymes. The pH of the culture medium can influence microbial growth, and induce the expression of genes that result in changes in phenotypes, such as changes in morphology, physiology or the expression of enzymes. The limitation of growth has been associated with a reduction in the production and/or activity of extracellular enzymes (Madigan et al., 2004). Figures 6 and 7 shows the stability of the enzyme regarding the incubation at different pH values and temperatures, respectively.

In the more extreme pH values tested, *α*-amylase underwent a reduction in enzyme activity of at least 24%, with it being completely inhibited at pH 8.5. In the 6.0 - 7.5 pH range, the enzyme proved to be quite stable, maintaining at least 95% of its activity, with this being the recommended pH range for the application of the enzyme.

Thermal resistance is considered one of the most important criteria for the industrial application of the enzyme, given that most processes require the use of

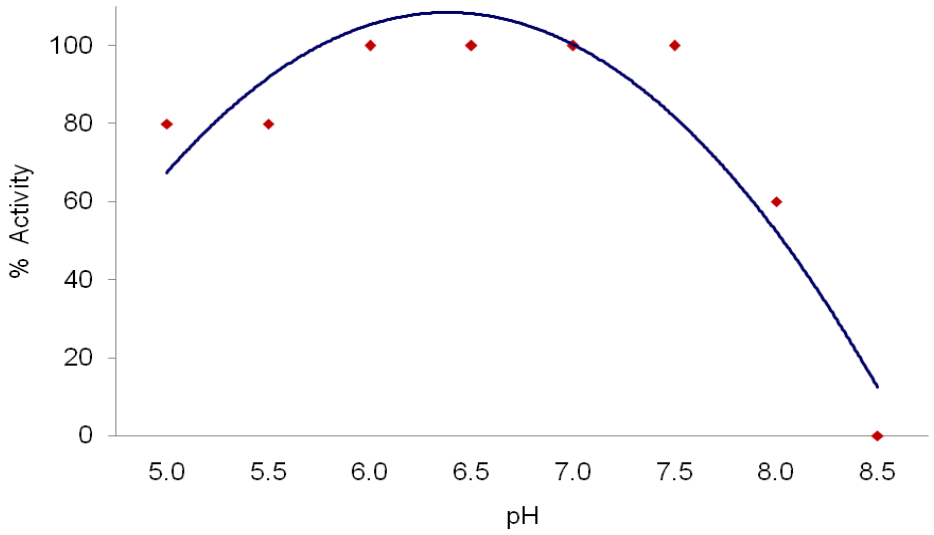

Figure 6. Stability of α-Amylase regarding incubation at different pH values.

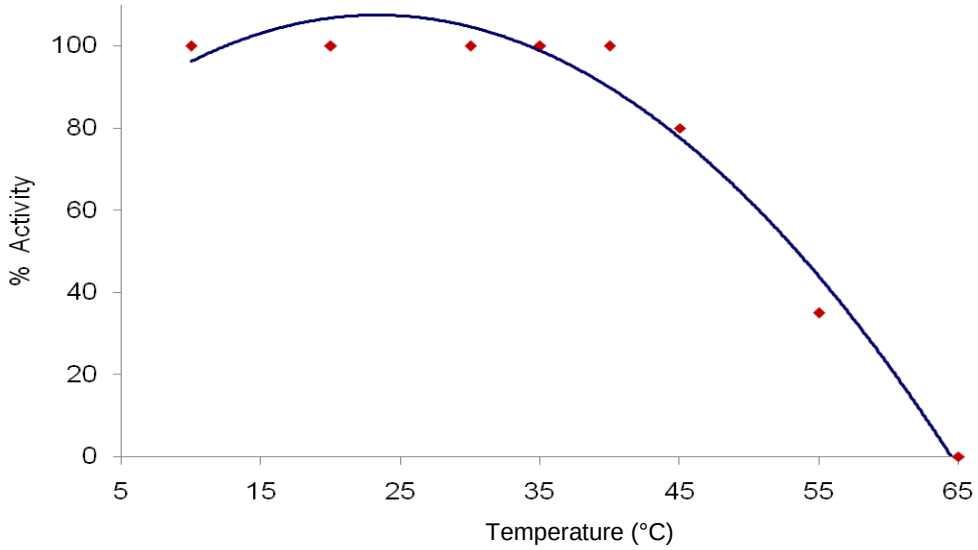

Figure 7. Stability of α-amylase regarding different incubation temperatures.

high temperatures (Ray and Nanda, 1996). The use of high temperatures for the activity of these enzymes, however, depends on the stability limit of the protein, since it will have a range within which its structure is maintained. Outside of this range, denaturation would occur, resulting in a loss of activity. An increase in the use of the α--amylase enzyme in biotechnology has occurred due to its extensive range of working conditions, including high temperatures, extreme pH, and the presence of surfactants and organic solvents (Tanaka and Hoshino, 2002).

When the enzyme was incubated at temperatures of 10 to 37°C, it preserved its full activity. When a temperature of 45°C was employed, the enzyme suffered a decline in residual activity, although this has been its optimal temperature of operation, indicating that the produced *α*-amylase should be applied at temperatures below 45°C to maintain its activity for a longer period of time.

Rasiah and Rehm (2009) obtained amylase which proved quite stable at 85°C. Figueira et al. (2000) produced amylases with the fungi *Fusarium moniliforme* and *A. flavus,* obtaining thermal stability close to 20°C. Fungal amylases are more sensitive to the elevation of temperature, tending to suffer a drastic drop in activity at temperatures above 50°C (Spier, 2005). This behavior was observed and is represented in Figure 6.

Understanding the thermo-stability of enzymes will promote their addition to products that need to go through some form of heat treatment during their processing, while preventing inactivation or with the intentional inactivation of the enzyme when the expected result of its catalytic activity has been obtained.

Conclusion

With the results obtained, it is possible to conclude that the production of α-amylase through SSF was more efficient than the production of the enzyme through SmF, obtaining more than twice the activity found in SmF with SSF, with values of 1625 and 712 U/mL for SSF and SmF, respectively. At 120 h of solid state fermentation using rice bran as substrate, 1625 U/mL was obtained, demonstrating the excellent α-amylase secretion ability of the D1-FB strain of the endophytic fungus, *P. digitatum*, isolated from *B. dracunculifolia* D.C. (Asteraceae). The enzyme characterization tests revealed an optimal pH at 6.5 and an optimal temperature at 45°C. The α-amylase produced was stable in the neutral pH range, but it showed great drops in activity with temperatures above 45°C and in the presence of ions at a concentration of 5 mM. These results allow for the conclusion that the produced α-amylase may be a new enzyme alternative for application in industries that require the saccharification of starch.

Conflict of interests

The authors have not declared any conflicts of interests.

REFERENCES

Bernhardsdotter ECMJ, Nig JD, Garriott OK, Pusey ML (2005). Enzymic properties of an alkaline chelator-resistant a-amylase from an alkaliphilic *Bacillus* sp. isolate L1711. Process Biochem. 40:2401-2408.

Brena BM, Pazos C, Franco-Fraguas L, Batista-Vieira F (1996). Chromatographic methods for amylases. J. Chromatogr. 684:217-237.

Corradini E, Lotti C, Medeiros ES, Carvalho AJF, Curvelo AAS, Mattoso LHC (2005). Estudo comparativo de amidos termoplásticos derivados do milho com diferentes teores de amilose. Polímeros 15:268-273.

Del Bianchi VL, Moraes IO, Capalbo DMF (2001). Fermentação em estado sólido. In: Lima, U.A. et al. (Coord.). Biotecnologia Industrial: Engenharia Bioquímica. 1. Ed., v. 3. São Paulo: Edgard Blucher. 13:247-276.

Driss D, Zouari-Ellouzi S, Chaari F, Kallel F, Ghazala I, Bouaziz F, Ghorbel R, Chaabouni SE (2014). Production and *in vitro* evaluation of xylooligosaccharides generated from corncobs using immobilized *Penicillium occitanis* xylanase. J. Mol. Catal. B Enzym. 102:146-153.

Fernandes LP, Ulhoa CJ, Asquieri ER, Monteiro VN (2007). Produção de amilases pelo fungo *Macrophomina phaseolina*. Revista Eletrônica de Farmácia 4(1):43-51.

Fernandes MLM (2006). Produção de lipases por fermentação no estado sólido e sua utilização em biocatálise**.** 2006. 130f.

Dissertação (Doutorado em Química) - Setor de Ciências Exatas - Universidade Federal do Paraná. Curitiba, Brasil.

Figueira ELZ, Sá MC, Ida EI, Hirooka EY (2000). Produção e caracterização de amilases de *Fusarium moniliforme* e *Aspergillus flavus*. Bol. Centro Pesqui. Process Aliment. 18(1):13-26.

Germano S (2000). Desenvolvimento de bioprocessos para produção, caracterização e purificação de proteases de *Penicillium* sp. por fermentação no estado sólido. 2000. 142f. Tese (Doutorado em Processos Biotecnológicos) - Setor de Tecnologia - Universidade Federal do Paraná. Curitiba, Paraná, Brasil.

Giongo JL (2006). Caracterização e aplicação de proteases produzidas por linhagens de *Bacillus sp*. 2006. 95f. Dissertação (Mestrado em Microbiologia Agrícola e do Ambiente) –Faculdade de Agronomia – Universidade Federal do Rio Grande do Sul. Porto Alegre, Rio Grande do Sul, Brasil.

Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B (2003). Microbial α-amylases: A biotechnological perspective. Process Biochem. 38(11):1599-1616.

Hashim SO, Delgado OD, Martinez MA, Kaul RH, Francis JM, Mattiasson B (2005). Alkaline active maltohexaose-forming α-amylase from *Bacillus halodurans* LBK 34. Enzyme Microb. Technol. 36:139-146.

Hattori T, Kato Y, Uno S, Usui T (2013). Mode of action of a β-(1→6)-glucanase from *Penicillium multicolor*. Carbohydr. Res. 366:6-16.

Hu Y, Liu G, Li Z, Qin Y, Qu Y, Song X (2013). G protein-cAMP signaling pathway mediated by PGA3 plays different roles in regulating the expressions of amylases and cellulases in *Penicillium decumbens.* Fungal Genet. Biol. 58(59):62-70.

Lei Y, Liu G, Li Z, Gao L, Qin Y, Qu Y (2014). Functional characterization of protein kinase CK2 regulatory subunits regulating *Penicillium oxalicum* asexual development and hydrolytic enzyme production. Fungal Genet. Biol. 66:44-53.

Lorentz RH (2005). Seleção de isolados de Paenibacillus spp com atividade enzimática e antimicrobiana. 2005. 103f. Dissertaç,o (Mestrado em Microbiologia Agricola e do Ambiente) –Faculdade de Agronomia –Universidade Federal do Rio Grande do Sul. Porto Alegre, Rio Grande do Sul, Brasil.

Madigan MT, Martinko JM, Parker J (2004). Microbiologia de Brock. 10. ed. Sao Paulo: Person Education. 517p.

Michelin M (2005). Estudo da glucoamilase e da α-amilase produzidas pelo fungo Paecilomyces variotii: Purificaçao, caracterizaçao bioqu,mica e relaçės filogenéticas. 2005. 160f. Dissertaçao (Mestrado em Ciências) –Departamento de Biologia –Universidade de Sao Paulo, Ribeirao Preto, Brasil.

Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31(3):426-428.

Mitchell DA, Lonsane BK (1992). Definition, characterization and economic evaluation. In. Solid Substrate Cultivation, Elsevier, London.

Moraes LMP (2004). Amilases. In. Said, S.; Pietro, R. Enzimas como agentes Biotecnolģicos. Ribeirao Preto: Legis Summa. pp. 123-128.

Myers AM, Morell MK, James MG, Ball SG (2000). Recent progress toward understanding biosynthesis of the amylopectin crystal. Plant Physiol. 122:989-997.

Najafi MF, Deobagkar D, Deobagkar D (2005). Purification and characterization of an extracellular α-amylase from *Bacillus subtilis* AX20. Protein Expr. Purif. 41:349-354.

Oliveira AN, Flor NS, Oliveira LA (2010). Influência do pH e temperatura sobre a atividade amilolitica de rizbios isolados de solos da Amazônia. Acta Amaz. 40(2):401-404.

Oliveira AN, Oliveira LA, Andrade JS, Chagas-Junior AF (2007). Produçao de amilase por rizbios, usando farinha de pupunha como substrato. Ciênc. Tecnol. Aliment. 27(1):61-66.

Onofre SB, Groff SA, Sartori A, Bertolini J, Kagimura FY, Rotta D, Mazzali L, Steilmann P (2011). Amylolytic Enzymes Produced by the Fungus *Colletotrichum gloeosporioides* in Rice Semi-Solid Fermentation. J. Yeast Fungal Res. 2:28-32.

Onofre SB, Groff SA, Sartori A, Bertolini J, Kagimura FY, Rotta D, Mazzali L, Steilmann P (2012). Production of *α*-Amylase and Amyloglucosidase by the Fungus *Cylindrocladium* sp. in Semi-Solid State Fermentation. J. Microbiol. Res. 2:123-126.

Pandey A (2002). Solid state fermentation. Biochem. Eng. J. 3636:1-4.

Pandey A, Soccol CR, Rodrigues-Leon JA, Nigam P (2001). Solid state fermentation in biotechnology. Nova Deli, Asiatech. P 221.

Pandey A, Webb C, Soccol CR, Larroche C (2005). Enzyme Technology. 1st ed. New Delhi: Asiatech Publishers, Inc., P 760.

Paris LD (2008). Produçao de enzimas fngicas por fermentaçao em estado slido das sojas orgânica, transgênica e convencional. 115p. 2008. Dissertaçao de Mestrado. Programa de Ps-graduaç,o em Engenharia Quamica, Centro de Ciências Exatas, Universidade Estadual do Oeste do Parana (Unioeste), Toledo, Parana, Brasil.

Rasiah IA, Rehm BHA (2009). One-step production of immobilized α-amylase in recombinant *Escherichia coli*. Appl. Environ. Microbiol. 75(7):2012-2016.

Ray RR, Nanda G (1996). Microbial β-amylases: Biosynthesis, characteristics, and industrial applications. Crit. Rev. Microbiol. 22:181-199.

Rocha CP (2010). Otimizaç,o da Produç,o de Enzimas por *Aspergillus niger* em Fermentaçao em Estado Slido. 136p. Dissertaçao de Mestrado. Programa de ps-graduaç,o em Engenharia Quamica, Universidade Federal de Uberlândia, Faculdade de Engenharia Quamica, Uberlândia, Brasil.

Silva ARZ (2002). Desenvolvimento de bioprocessos para a produçao de fitase por *Aspergillus niger* em fermentaçao no estado slido utilizando subprodutos agracolas para aplicaçao como aditivo na alimentaçao de aves e suanos. 2002. 96f. Dissertaçao (Mestrado em Tecnologia de Alimentos) –Setor de Tecnologia –Universidade Federal do Parana. Curitiba, Brasil.

Soccol CR (1992). Physiologie et Métabolisme de *Rhizopus* em Culture Solide et Submergée em Relation Avec la Dégradation d'Amidon et la Production d'Acide L(+) Lactique. 218p. Thése de Doctorat. Mention Génie enzymatique, Bioconversion et Microbiologie, Université de Tecnologie de Campiègne. Compiègne-France.

Spier MR (2005). Produçao de enzimas amilol,ticas fngicas α-amilase e amiloglucosidase por fermentaçao no estado slido. 2005. 178f. Dissertaçao (Mestrado em Tecnologia de Alimentos) –Setor de Tecnologia –Universidade Federal do Parana. Curitiba, Brasil.

Tanaka A, Hoshino E (2002). Thermodynamic and activation parameters for the hydrolysis of amylose with *Bacillus* α-amylases in a diluted anionic surfactant solution. J. Biosci. Bioeng. 93:485-490.

Thys RCS (2004). Produçao, caracterizaçao, purificaçao e aplicaçao de uma protease produzida pelo microrganismo *Microbacterium* sp. *kr10*. 2004. 114f. Dissertaçao (Mestrado em Microbiologia Agracola e do Ambiente) –Faculdade de Agronomia –Universidade Federal do Rio Grande do Sul. Porto Alegre, Brasil.

Tomazic SJ (1991). Protein stabilization. In. Biocatalysts for industry. Topic in Applied Chemistry. Ed. Plenum Press, New York and London. 330p.

Yang CH, Liu WH (2014). Purification and properties of a maltotriose producing α-amylase from *Thermobifida fusca.* Enzyme Microb. Technol. 35:254-260.

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Full Length Research Paper

Development of bio-hybrid material based on *Salmonella* Typhimurium and layered double hydroxides

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The immobilization of a whole microbial cell is an important process used in nanotechnology of biosensors and other related fields, especially the development of bio-hybrid materials based on live organisms and inorganic compounds. Here, we described an essay to develop a bio-hybrid material based on *Salmonella* Typhimurium cells and layered double hydroxides (LDH). The synthetic clays have a good capacity to be a host matrix for immobilization of live entity like bacteria. The incorporation of LDH in the nutritive broth shows the capacity of bacteria to grow under the inorganic conditions. The immobilization of bacteria onto the LDH Layer deposited on gold wafers was successfully done and the verification of the final material consistence was given by Fourier transform infrared spectroscopy (FTIR) analysis that shows the possibility of various covalent links that can be established between the polar functional group of the cell and the interlayer level in the LDH. The roughness of the surface was given by scanning electron microscope (SEM) imaging and shows the homogeneity of cell distribution on the LDH layer.

Key words: Layer double hydroxide, *Salmonella* Typhimurium, Fourier transform infrared spectroscopy-attenuated total reflectance (FTIR-ATR), X-ray diffraction (XRD), energy dispersive X-ray (EDX); scanning electron microscope (SEM).

INTRODUCTION

The immobilization of enzymes for catalytic reactions coupled with redox reactions has gained an immense importance during the last two decades in the world of biosensors. Development of biosensor requires the purification of biomolecules, which is a sensitive technique and requires several critical conditions, that is

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why the investigation using these materials was left only in the research laboratories and does not rise *in situ*. Thus, the immobilization of living entities such as bacteria reduces the strokes of purification and the several conditions necessary for the bioactive layers. Bacteria can be used like bio-recognition component in biosensors to investigate the environmental toxicity caused by various media in the case of soil, sediment and water. The coupling of bacteria with device transducer can convert a cellular response into detectable signals and make it possible to exploit the sensitivity of the cell to toxins and pollutants (Hua et al., 2015). By immobilized technique, the cell can retain most of its functionality and a wide range of enzymes will be protected in the cell, which can be exploited in several applications like detection or use for the synthesis of some substances for other applications, which can then react with substrates outside the cell wall. Present as free forms, biomolecules such as enzymes, antibodies and receptors, presented in microorganisms as well as animal and plant cells or tissues have been used as biological sensing elements. Among these, microorganisms offer advantages of ability to detect a wide range of chemical substances (Banerjee et al., 2013).

Considerable attention has been given to native biomolecules in the microorganisms which conserve these biomolecules naturally and can easily modify them on large scale at the same time. It is better to keep the biomolecules solicited in their native medium which is the best method to preserve the sensitivity after the exploration of this propriety which will be seen as the ability of the whole cell to be immobilized and manipulated.

In our work, we studied the interaction between layered double hydroxides (LDHs) layer and bacterial cell. LDHs can offer various advantages such as the capability to be an interest host matrix for a guest bacteria cell and it makes it easy to study some external molecules and bacterial comportments in this new environment. LDHs can be represented by the general formula: $M^{2+}_{1-x}M^{3+}_{x}(OH)_2\ A^{n-}_{x/n}\ mH_2O$ (Ayawei et al., 2015a), M^{2+} are divalent cations (Mg^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+}), M^{3+} are trivalent cations (Al^{3+}, Cr^{3+} and Fe^{3+}) and A^{n-} is interlayer anions (Cl^-, NO^-_3 , CO^{2-}_3 and SO^{2-}_4) balancing the charge on the layers (Rezvani et al., 2014). These layers have received a great attention due to their versatile properties which are result of their high ion exchange capacity (Balcomb et al., 2015) they also have various applications such as catalysis, (Fan et al., 2014; Ayawei et al., 2015b) gene and molecular reservoir (Zhang et al., 2014) and thin films (Vlada et al., 2014).

The objective of this work was to describe the influence and interaction of low LDH on the viability of various solutions of different concentrations and the survival of *Salmonella* Typhimurium deposed on thin layer of LDH. This bio-hybrid material was checked on structural level that was confirmed by consistency of interfaces and stability of the bacteria under the LDH materials.

MATERIALS AND METHODS

Wafer device and pre-treatment

The substrate sample used in this work was 0.25 × 0.25 cm that contained a thin gold layer deposited by LPCVD (100 nm) on an Insulator/Semiconductor (IS) substrate; wafers were graciously offered by Dra Campas M., laboratory of Biosensor IRTA-Sant Carlos de la Rapita (Spain). The samples were leached with compressed nitrogen and then rinsed with MQ before being incubated for one and half hour in sulfochromic acid solution, then rinsed with water and dried with nitrogen air.

Cell culture and preparation of samples

The microbiological experiments described in this paper were carried out with *Salmonella enterica* serovar Typhimurium strain SL1344 supplied by the professor Casadesus J. (Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Seville 41080, Spain). Bacteria were incubated in nutrient broth (NB) (Pronadisa, Spain) at 37°C overnight with shaking under aerobic conditions. An exponential bacterial culture was then centrifuged at 10,000 *g* for 15 min and washed twice in sterile phosphate-buffered saline (PBS) solution and suspended in the same buffer containing LDH (at a concentration of 5, 10 and 15×10^{-3} g mL^{-1}). In parallel, control samples were prepared in PBS. Treatments were carried out at 37°C without shaking. Samples were extracted at various times (0, 3 and 6 h), diluted as needed and spread on Nutrient-agar plates then incubated for one night at 37°C. Bacteria were then counted on every plate for three repetitions, the curves of viability and survival were drawn as log (N / N_0) according to time.

Preparation of LDHs

LDHs were prepared by co-precipitation method. As per the protocol developed by Vlada et al. (2014), 50 ml of aqueous of 0.8 M $MgCl_2$ and 0.4 M $AlCl_3$ or $FeCl_3$ solution was added dropwise to 200 mL of 0.4 M sodium carbonate solution. The pH of mixture was held constant at 9.6 for MgAl-LDH and 11 for MgFe-LDH by simultaneous addition of 1 M sodium hydroxide solution (Sampieri et al., 2011). The addition of the salt solution was completed in 4 h. The precipitate was matured for 24 h at room temperature, the final solution was filtered and then washed by desionized water. The LDHs was dried at 100°C for 24 h which was followed by crushing and characterization with XRD.

Characterization technique

Powder X-ray diffraction (XRD) analysis was performed on a Panalytical X'Pert Pro diffractometer using Co K1 (40 kV, 30 mA) radiation and continued scanning mode. The diffraction patterns were recorded in a 2 theta range from 7° to 80°, in steps of 0.16° and counting time of 2 s per step.

Absorbing Infrared Fourier Transformed (IRTF) spectra in ATR mode were recorded using QUINOX 55 (Bruker) spectrophotometer in the range of 4000 – 550 cm^{1} with 2 cm^{-1} resolution and averaging 64 scans. Imaging is loaded with scanning mode using an FEI-Quanta 200 Environmental Scanning Electron Microscope.

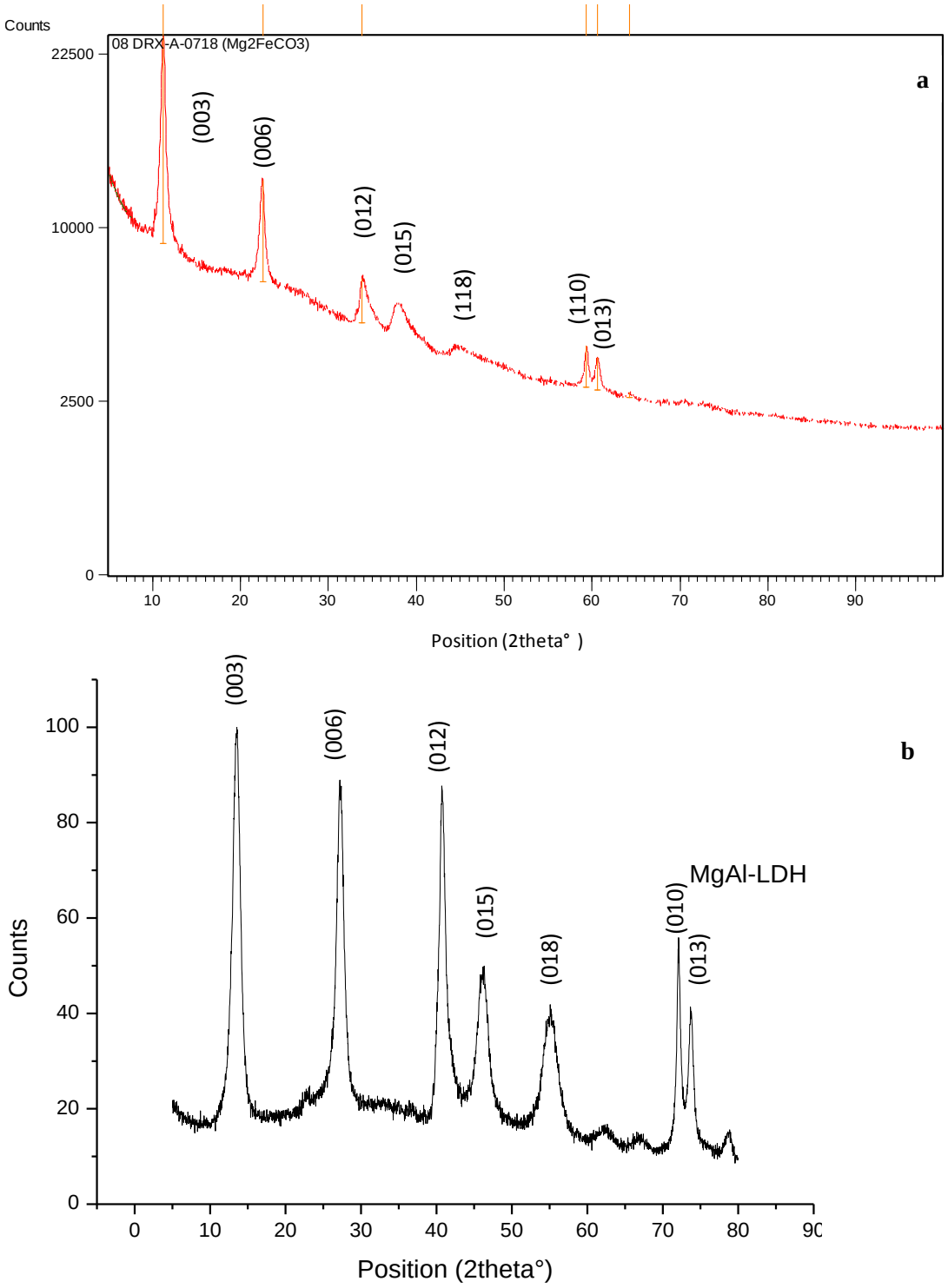


Figure 1. XR-diffractograms of Mg_4Fe (a) and Mg_2Al (b) LDHs prepared by co-precipitation.

RESULTS AND DISCUSSION

XRD characterization

The crystallinity of synthesized LDHs was analyzed with x-ray diffraction as function of weight percent inorganic component. Figure 1a and b shows x-ray diffraction pattern of various synthesized LDHs. Diffraction of $Mg_2Al(CO_3)_{0.5}(OH)_6$ *Rhomboedric R3/m* have a broad peak (006) at about $2\Theta = 25.92°$, which is a characteristic

Table 1. Comparative view based on indexed hkl plans.

hkl plans	2Θ (°) MgAl	2Θ (°) MgFe
(003)	15	12
(006)	28	23
(012)	42	34
(015)	47	38.5
(018)	55	-
(118)	-	45
(010)	72	-
(110)	-	59
(013)	75	61

Table 2. Crystalline parameters of synthesized LDHs.

Parameter	a(Å)	b(Å)	c(Å)	d(Å)
Mg_2Al	3.0	-	22.8	7.6
Mg_4Fe	3.13	3.13	15.66	-

peak of hydrotalcites (Sampieri et al., 2011; Bankauskaite and Baltakys, 2011). Studies on XRD patterns of $Mg_4Fe(OH)_{10}Cl(H_2O)3$ (Peng et al., 2014) hexagonal show all characteristic peaks of Irenty-HDLs and verifies that the crystallinity is symmetric (Dias et al., 2014).

To reveal the hkl-plans inventory of the two studied LDH, Table 1 shows many LDH characteristic peaks especially (003), (006) and (012) which are different only with the position. Many other peaks were absent and/or shifted; (018) and (010) appears respectively at 2Θ equal to 55° and 72° in the case of MgAl and does not appear in the case of MgFe, such movement in these peaks is governed by the crystalline system *Rhomboedric R3/m* in the first and *Hexagonal* in the second. However, the newest peaks appears in the case of MgFe at 45 and 59° of 2Θ position indexed to (118) and (110) respectively which can give the idea about the interlayer space and distance. To better explain the difference between the two LDH, elementary analysis have been done and the result are given in Table 2 which summarizes the crystalline parameter and confirms that the preparation of the LDH with co-precipitation synthesis method was successfully done according the study given by Hidouri et al. (2011), Abdelkader et al. (2011), Hidouri et al. (2011) and Abdelkader et al. (2011).

The value of the parameter *a* in case of MgAl was less than that of MgFe because the crystal radius of Al^{3+} was lower than that of Fe^{3+} (Chen et al., 2012; Lin et al., 2014). Because the type of M^{2+} is the same in the case of the two tested LDH, the parameter *c* value appears to be more dependent on the type of the intercalated anions CO_3 in the case of MgAl and Cl in the case of MgFe LDH.

The distinct reflections in the LDHs patterns given in Figure 1 and summarized in Table 1 indicate the formation of the typical lamellae structure and distinguish well the structure related to Al^{3+} and Fe^{3+} representing the M^{3+} of the LDH, and the intercalated ions. The details of crystalline phase given in Table 2 shows the value of the parameters of the related crystal and show the right obtained system which proved the success of the LDH synthesis using the co-precipitation method.

Capacity of bacteria to live in LDHs environment

The growth of bacteria was determined by estimation of the CFU of the suspensions at t = 0, 3 and 6 h. The analysis compared by the curves of viability of the bacterial suspensions witness and treated by synthetic clays show relative differences according to the LDH used (Figure 2). It is clear that the most important modifications got the bacterial suspensions brooded with MgAl, while the least important was recorded with MgFe.

The analysis of the curves of viability shows that the addition of the LDH practically has no considerable effect on the growth for a concentration equal to 1 and 5.10^{-3} g mL^{-1} added in the flask. However, a remarkable difference is noted for the 15×10^{-3} g mL^{-1} added in the culture; the effect becomes more noticeable for the LDH incorporate magnesium divalent metal (Figure 2a). The remarkable effect of the MgFe can take place for high concentration equal to 15×10^{-3}g mL^{-1}. The capacity of bacteria to survive in the PBS buffer doped with LDH seems more important when the concentration in LDH increases; the presence of LDHs in the medium do not have any inhibitive effect on growth of bacteria (Figure 2b), however, it appears that at this concentration the LDH has a stimulation effect in the case of 15×10^{-3} g mL^{-1} of MgAl. In the next part, bacteria was used as a model to study the relationship of *Salmonella* cells deposited on thin film of LDH, the biomaterial have been deposited on gold wafers, several techniques were used to conclude on the presence of the life entity on the surface of the inorganic compound.

The absence of inhibition of the growth of bacteria in the case of MgAl-LDH is probably due to ionization of CO_3 compound of LDH in the salt solution (Yao et al., 2015; Halma et al., 2015) that makes accessible source of carbon and enables bacteria to survive in this case. This effect is not seen in the case of LDH with MgFe because the intercalated ion was the chloride, herein the plausible hypothesis to explain this difference between LDH based on MgFe and LDH based MgAl, that MgFe is more stable and bacteria cannot use any compound of the structure which also proves that the bactericidal effect of chloride inhibits bacteria growth in this case which is comparable with the growth in the PBS solution. The conclusion relates to the hypothesis the LDH suspension conserves *Salmonella* Typhimurium growth and it depends on the intercalated ion in the LDH.

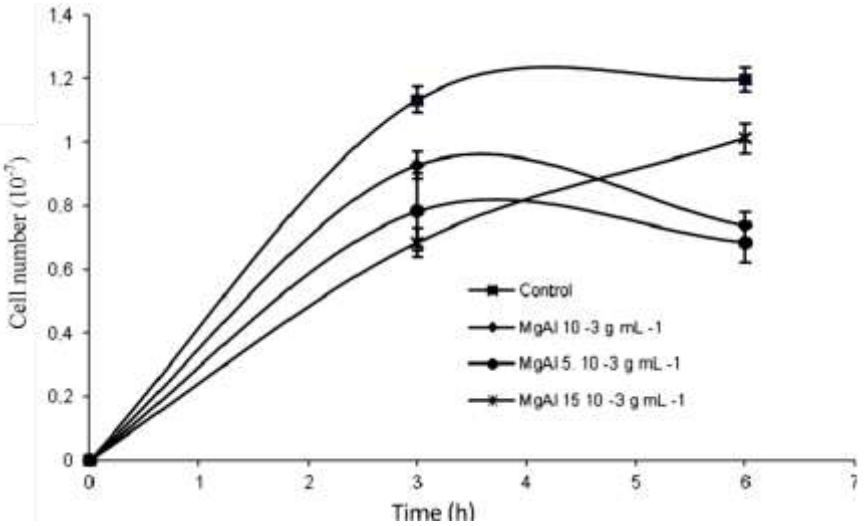


Figure 2a. Curves of viability test.

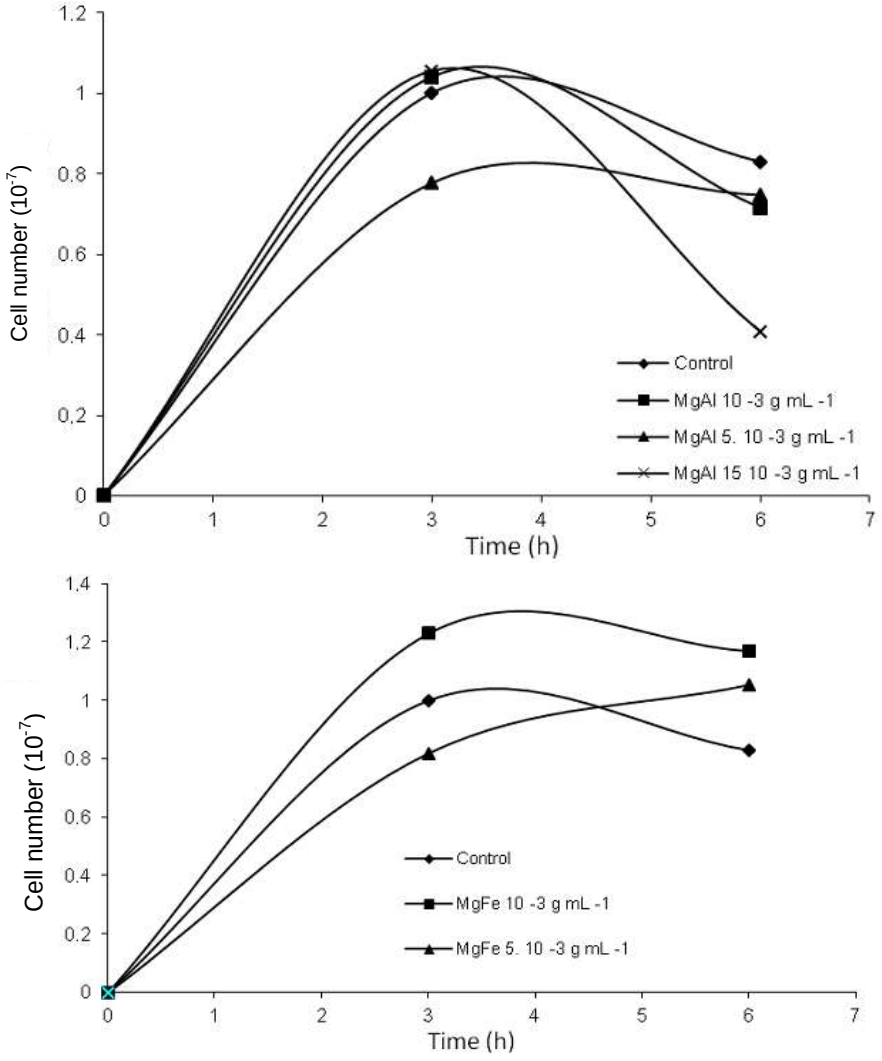


Figure 2b. Curves of viability test of bacteria in the PBS solution doped with various LDHs.

Bacterial finger on LDHs matrix

The main goal in the immobilization of bacteria is to prove that on interface level, bacteria can interact with the host matrix. FTIR analysis given in Figure 3a and b show the interface consistency in the FTIR spectrum of hybrid bacterial/LDHs/Au (surface), which were represented by the percent of transmittance and plotted as a function of wavenumber (cm^{-1}). In the case of bacteria/MgFe/Au (surface), spectra were given in Figure 3a. The spectrum exhibit various picks that demonstrate the possibility of bacteria cells to establish various types of links with MgFe-LDH, at 1564-1512 and 1485-1390 cm^{-1} which are due to the presence of N-H links. The medium intensity bond observed at 1238 and 1235 cm^{-1} assigned to $(PO_2)^-$ asymmetric stretching modes of the phosphodiester indicated that extra-membranous phospholipids can interact with LDH, also the amide III/CH2 vibration proves the role of membranous protein that react on their side, which is shown by Touisni and collaborators (2013), when the amide III/CH2 comes from the glycine backbone and protein side chain (Touisni et al., 2013). The picks situated at 1000-890 cm^{-1} are due to the =C-H bond stretching vibration. The FTIR spectra of bio-hybrid material in the case of Mg_2AlCO_3 matrix exhibit various picks distinctly which are assigned to the presence of various hydroxide links. Figure 3b summarizes the essential peaks, the thiol links at 2600-2550 cm^{-1} in both LDH with aluminum and iron which makes it possible to conclude on the role of the polar function of amino acid to participate to entrap bacteria onto LDH profound structure with her hydroxide layer.

The infrared investigation illustrate that the interactions between bacteria and HDLs was established with anions of compensators charge layer of HDLs and the free amine, hydroxide and phospholipids from bacteria. The finger of bacteria into HDL matrix was made by polar functions of membrane proteins which are in general glycoprotein. It is notable that amine groups and hydroxide of membrane proteins interacts during 12 h with the thin coat layer of HDL. The presence of carbon-carbon bonds show that amino acid carbon has also the ability to interact. The sharp bonds observed at 1656 and at 1564 cm^{-1} are assigned to the C=O stretching vibration (amide I) and to the C-N stretching/N-H bending vibration (amide II) of the tissue proteins respectively (Baccar et al., 2011). The amide bond are primarily associated with the stretching action of the C=O group. This C=O bond may be due to peptide linkage and also depends on the protein's overall secondary structure (Hussein-Al Ali et al., 2012). The various covalent links given by FTIR suggest that there is a dynamic relationship between the life entity and inert materials given here by bacteria cells and LDH.

SEM imaging of bacteria land within LDH layer

The cartography of the synthesized bio-hybrid material based on hosts LDHs and bacteria with SEM is shown in Figures 4 and 5. Scanning images were obtained from a diluted solution of bacteria. The SEM images show the presence of bacteria in LDH deposited matrix, which are homogeneously distributed throughout the layer of LDH, which is also confirmed for all samples.

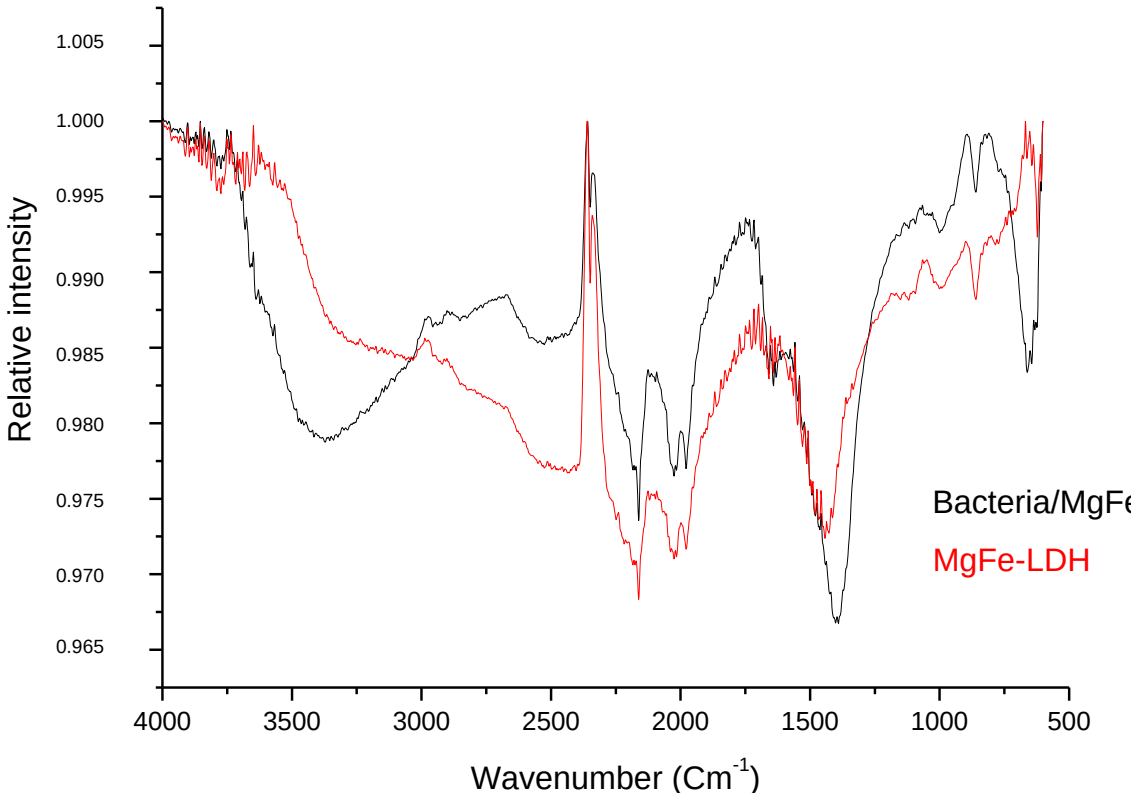
A drop deposition of viable cells of bacteria on HDLs thin layer promote oriented comportment, so that a deposited cell continues to divide and each cell produces a colony, as shown by the SEM images; there are visible areas where the density of bacteria was at the highest level, and other field still intact. It is also important to note that the area of bacteria existence confirms the results provided by the viability test; bacteria is not completely inhibited by the presence of HDL in its environment, and *Salmonella* have the ability to use HDL as a source of energy that can be carbon and/or magnesium. EDX analysis (Figure 4d and Figure 5e) confirms the chemical composition of the bacteria/LDH/Au (surface) bio-hybrid material for all samples and it was obtained at high density area of the surface. The EDX investigation show the presence of all compounds potentially present in the composite synthesized with bacteria, LDH and gold wafer.

LDH-bacteria interactions show a double capacity of *Salmonella* and LDH to be able to react with them to make one micro-hybrid material, stable and rich with various covalent links. This interaction can play an essential role in biological events, including membrane depending phenomena. In this context, SEM imaging explains various comportments of *Salmonella* with LDHs and makes clear a potentially biodegradation of LDH within *Salmonella*.

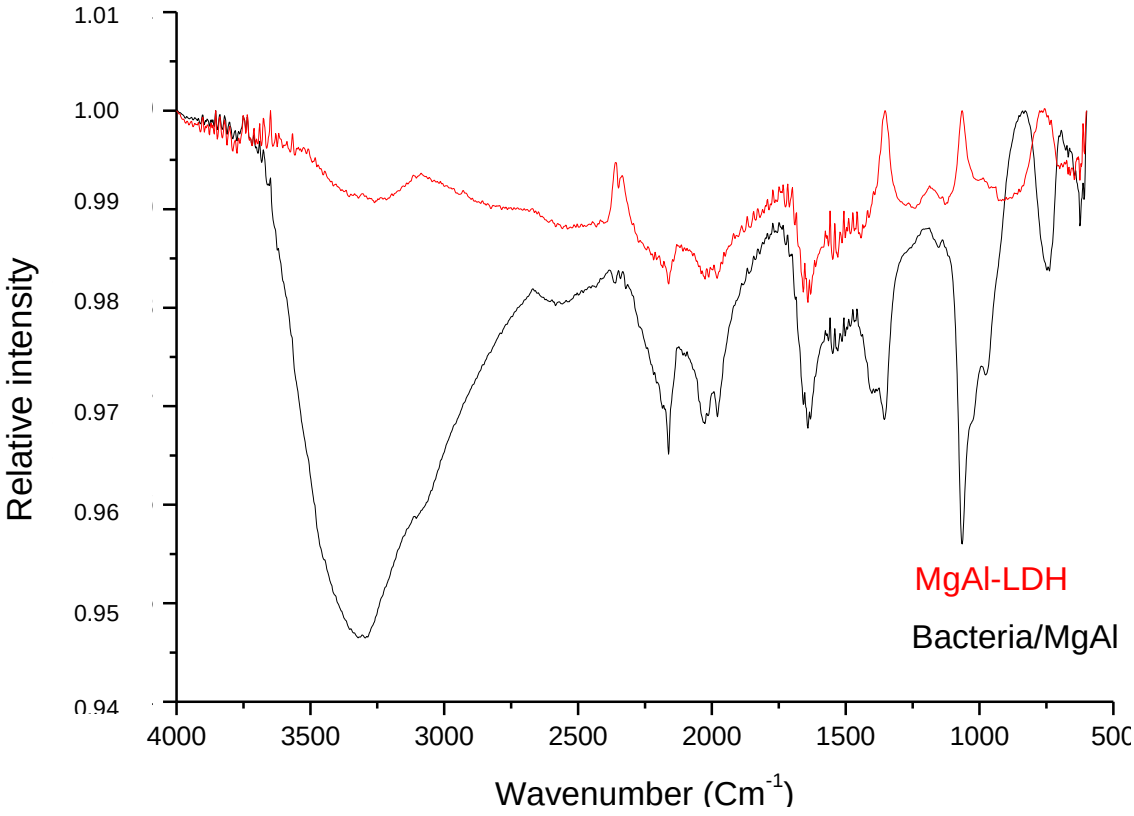
Bacteria adhesion or interaction with LDH layer is a key initiating step in nanobiotechnology based on whole cell and makes it easy to understand the process required to activate all layers that participated in the synthesized micro-hybrid materials. The interaction between *Salmonella* cells and LDH matrix makes a good profile of immobilization of the cell on inorganic compound and readjust the figure of the immobilization by inclusion. Bacteria conserves its activity, as reported by Bi et al. (2014) for biomolecules in that LDHs are a good reservoir for biomolecule for its ability to conserve the major functionality when they are immobilized on LDHs (Bi et al., 2014). It should be noted that the LDH can also be a good reservoir for bacteria cell and it conserves its biological ability and participate in bacteria cells to develop a micro-biofilm protection under LDH.

Conclusions

It is important to mention that the insignificance of differences observed in the survival of bacteria within LDHs and witness samples is given by the tolerance of *Salmonella* cells according the two tested LDH phases, MgAl and MgFe. The presence of bacterial layer in the



(a)



(b)

Figure 3. Infrared spectra of hybrid Salmonella/LDHs-Au. **(**a) Salmonella/MgFe-Au; (b) salmonella/MgAl-Au.

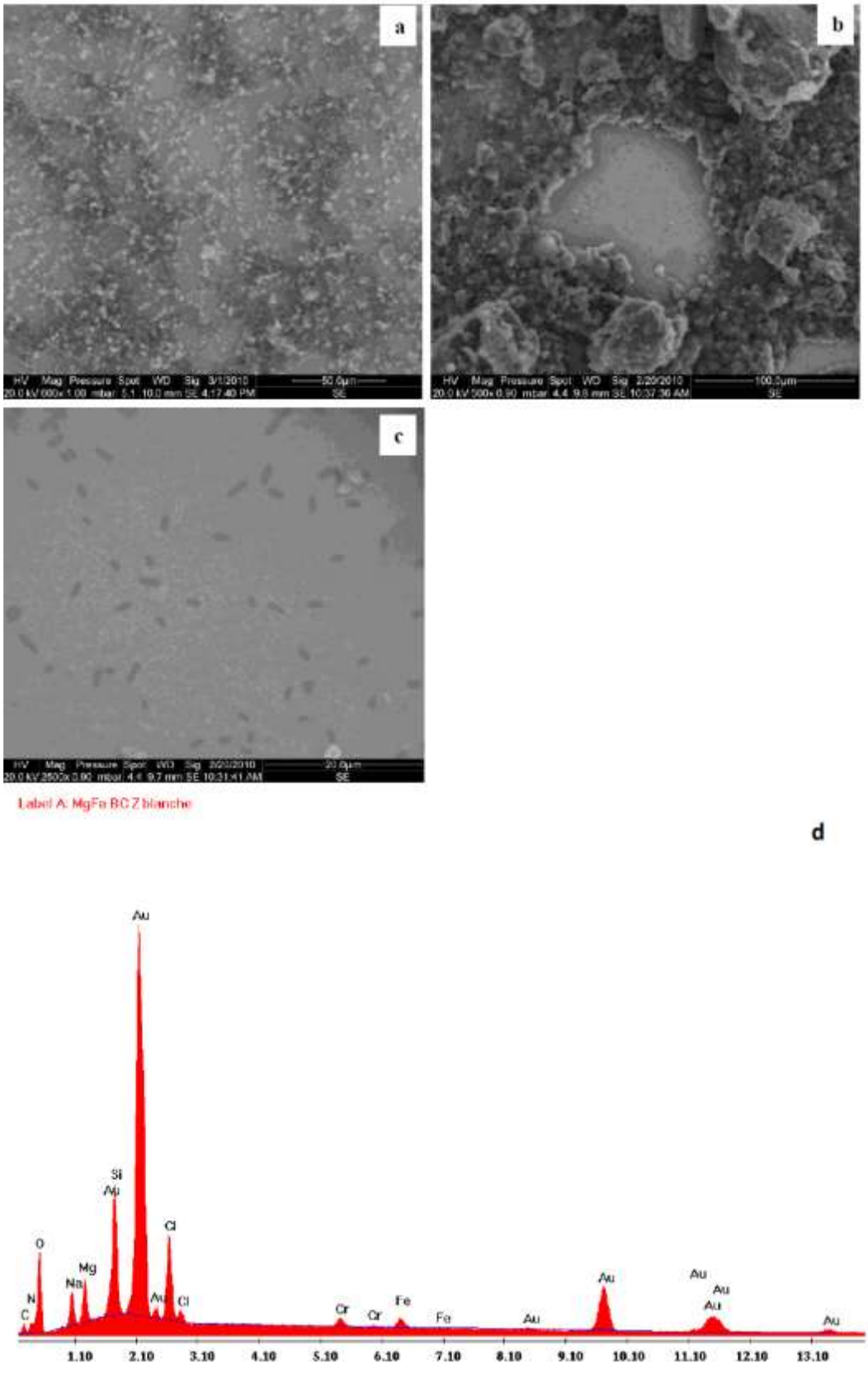


Figure 4. SEM imaging of deposited *Salmonella T*. within MgFe Layer. A. MgFe layer Scanning Image. B. Localized area with high density of bacteria within LDH layer. c. Salmonella T. in direct contact with the solid substrate of Gold. d. EDX analysis with the hybrid composite in cas of MgFe LDH.

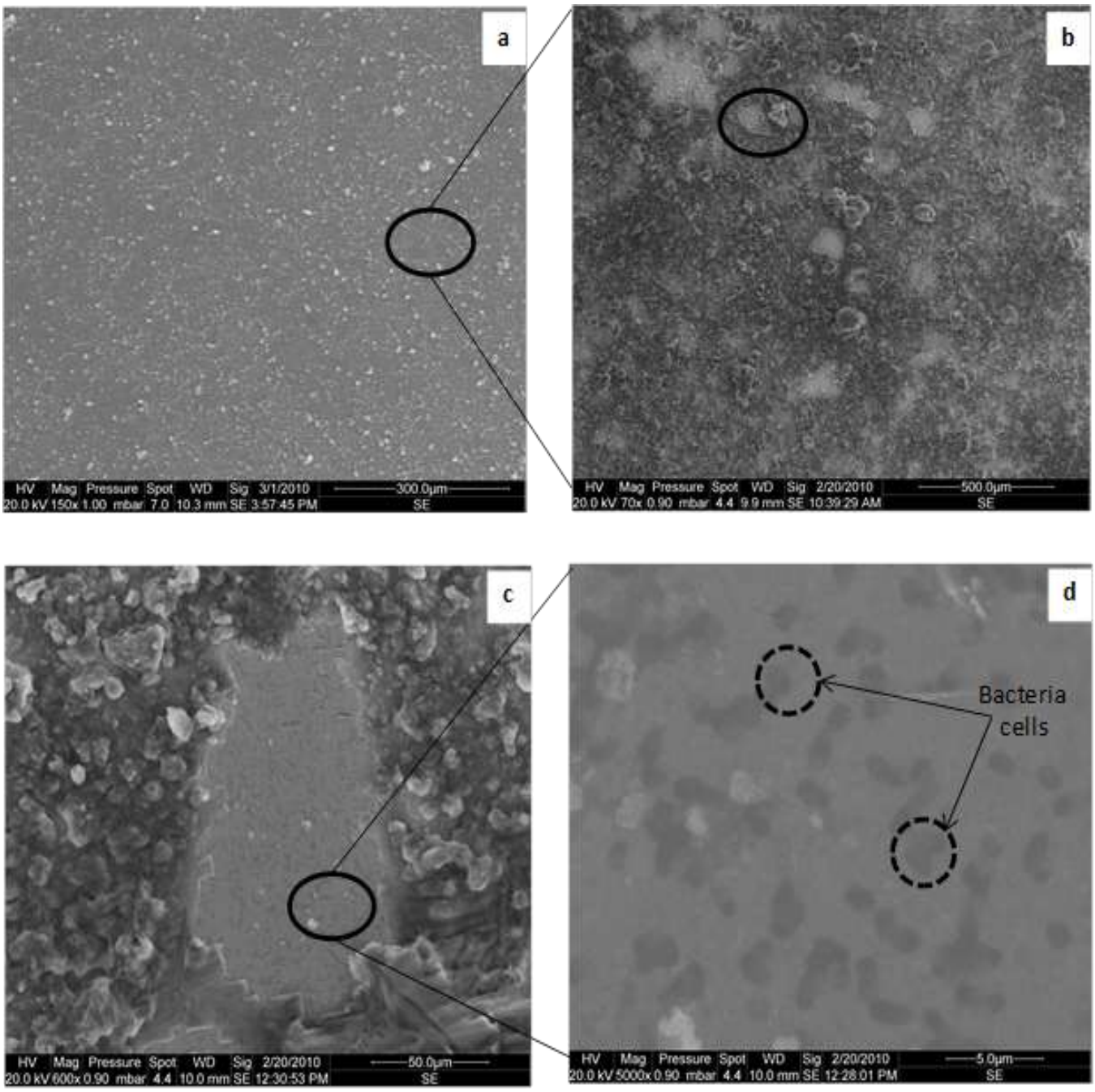


Figure 5. SEM Imaging of deposited *Salmonella* T. within MgAl Layer. (a) MgAl layer Scanning Image. (b) General vision with area of high density of bacteria within LDH layer. (c) Localized area with high density of bacteria within LDH layer. (d) *Salmonella* T. in direct contact with the solid substrate of Gold. (e) EDX analysis with the hybrid composite in the case of MgFe LDH.

integrated bio-hybrid material can offer an important advantage to biotechnology application that utilize periplasmic enzymes and more extended phospholipids and/or the extracellular polymeric substances that can catalyze reactions (Bruna et al., 2015); in this way, this bio-hybrid material can be useful for development of biosensors, the application desired in the beginning of this paper.

For the contact of bacteria cells with LDH, infrared analysis showed the presence of a wide range of possible covalent links established between LDH and viable cell of bacteria by the extra-cellular extensions like glycoproteins and phospholipids. The alteration takes place in proteins and phospholipids; it may be an adverse

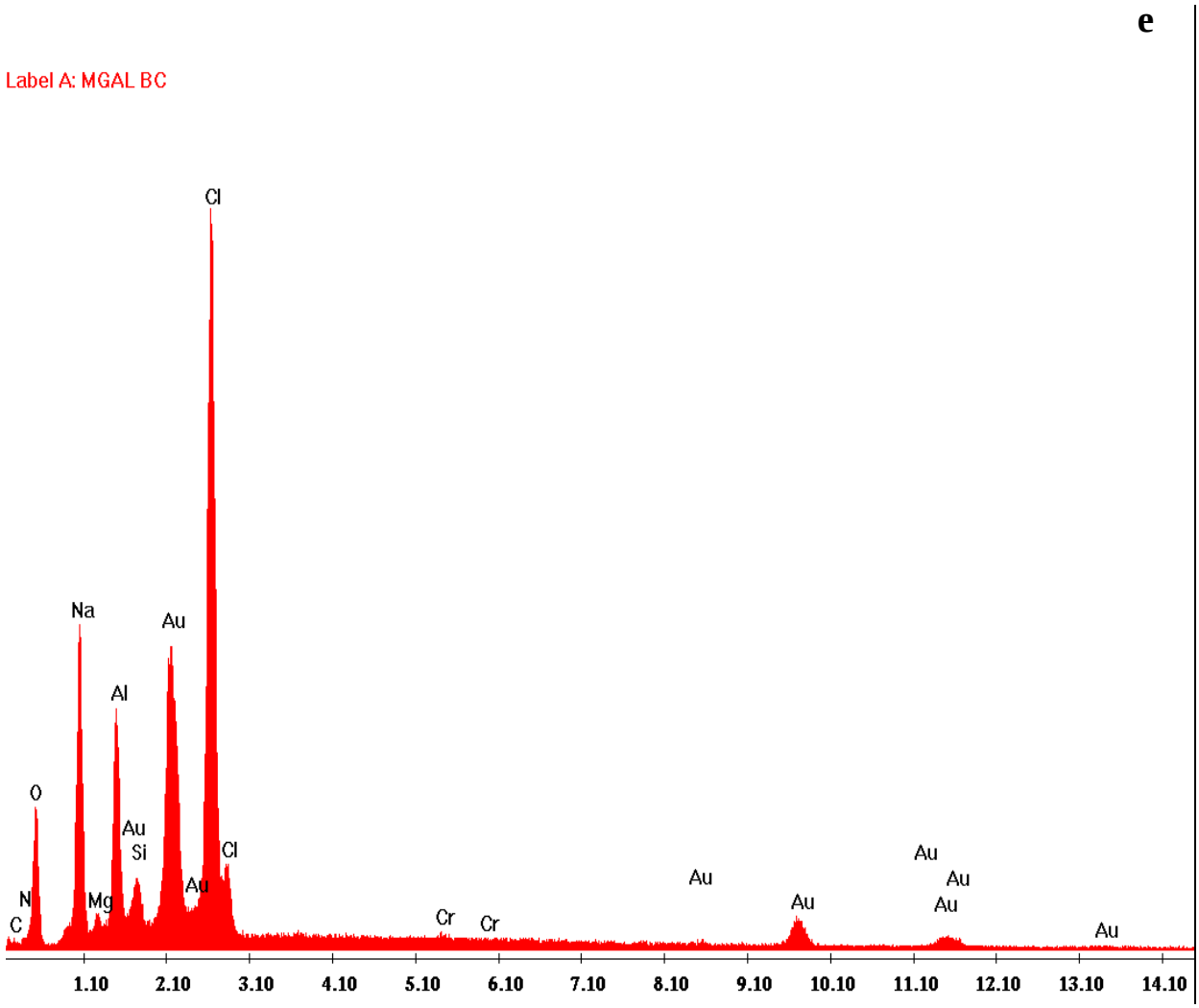


Figure 5. Contd.

effect on some important physiological processes, performing different biological events to maintain homeostasis of the cell. Therefore, the structural interactions can be considered as a good diagnostic tool carrying the effect of structural alteration caused by the covalent links on the functional processes of the bacteria. The work was done to study the feasibility of a bio-hydrid material obtained by deposition of viable *Salmonella* cells on gold wafer functionalized with LDHs and was tested at a structural side which show the good consistency and stability of the biomaterials that can be suitable for high technology applications, especially for development of biosensors and/or biopile based on immobilized live entities.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Abdelkader B-H N, Bentouami A, Derriche Z, Bettahar N, De Ménorval L-C (2011). Synthesis and characterization of Mg–Fe layer double hydroxides and its application on adsorption of Orange G from aqueous solution. Chem. Eng. J. 169:231-238.

Ayawei N, Angaye SS, Wankasi D, Dikio ED (2015a). Synthesis, Characterization and Application of Mg/Al Layered Double Hydroxide for the Degradation of Congo Red in Aqueous Solution. Open J. Phys. Chem. 5:56-70.

Ayawei N, Ekubo AT, Wankasi D, Dikio ED (2015b). Synthesis and Application of Layered Double Hydroxide for the removal of Copper in Wastewater. Int. J. Chem. 7(1):122-132.

Baccar ZM, Hidouri S, Errachid A, Ruiz Sanchez O (2011): Study of Bi-Enzyme Immobilization onto Layered Double Hydroxides Nanomaterials for Histamine Biosensor Application. J. Nanosci. Nanotechnol. 11(10):8798-803.

Balcomb B, Singh M, Singha S (2015): Synthesis and Characterization of Layered Double Hydroxides and Their Potential as Nonviral Gene Delivery Vehicles. Chem. Open 4(2):137-145.

Banerjee P, Kintzios S, Prabhakarpandian B (2013): Biotoxin Detection Using Cell-Based Sensors. Toxins 5:2366-2383.

Bankauskaite A, Baltakys K (2011). The Hydrothermal Synthesis of Hydrotalcite by Using Different Partially Soluble and Insoluble in

Water Mangesium and Aluminium Components. Sci. Sintering 43:261-275.
Bi X, Zhang H, Dou L (2014). Layered Double Hydroxide-Based Nanocarriers for Drug Delivery. Pharmaceutics 6(2):298-332.
Bruna F, Pereira MG, De Lourdes M, Polizeli TM, Valim JB (2015). Starch Biocatalyst Based on α‑Amylase-Mg/Al-Layered Double Hydroxide Nanohybrids, ACS Appl. Mater. Interfaces 7:18832-18842.
Chen C, Gunawan P, Lou XW, Xu R (2012). Silver Nanoparticles Deposited Layered Double Hydroxide Nanoporous Coatings with Excellent Antimicrobial Activities. Adv. Funct. Mater. 22(4):780-787.
Dias L, Cunha A, Vieira C (2011). Synthesis and properties of A6B2 (OH)16Cl24H2O (A = Mg, Ni, Zn, Co, Mn and B = Al, Fe) materials for environmental applications. Mater. Res. Bull. 46:1346-1351.
Fan G, Li F, Evans DG, Duan X, (2014): Catalytic applications of layered double hydroxides: recent advances and perspectives. Chem. Soc. Rev. 43:7040-7066.
Halma M, Mousty C, Forano C, Sancelme M, Besse-Hoggan P, Prevot V (2015). Bacteria encapsulated in layered double hydroxides: towards an efficient bionanohybrid for pollutant degradation. Colloids Surf. B Biointerfaces 126:344-50.
Hidouri S, Baccar ZM, Abdelmelek H, Noguer T, Marty J-L, Campàs M (2011). Structural and functional characterisation of a biohybrid material based on acetylcholinesterase and layered double hydroxides Talanta 85:1882-1887
Hua A, Gueuné H, Cregut M, Thouand G, Durand M-J (2015). Development of a bacterial bioassay for atrazine and cyanuric acid detection. Front. Microbiol. 6:211.
Hussein-Al Ali SH, Al-Qubaisi M, Hussein M Z, Ismail M, Zainal Z, Hakim MN (2012): Controlled release and angiotensin-converting enzyme inhibition properties of an antihypertensive drug based on a perindopril erbumine-layered double hydroxide nanocomposite. Int. J. Nanomed. 7:129-2141.
Lin Y, Fang Q, Chen B (2014). Metal composition of layered double hydroxides (LDHs) regulating ClO4- adsorption to calcined LDHs via the memory effect and hydrogen bonding. J. Environ. Sci. 26(3):493-501.
Peng F, Luo T, Yuan Y (2014). Controllable synthesis of Mg–Fe layered double hydroxide nanoplates with specific Mg/Fe ratios and their effect on adsorption of As (V) from water. New J. Chem. 38(9):4427-4433.
Rezvani Z, Khodam F, Mokhtari A, Nejati K (2014). Amine-Assisted Syntheses of Carbonate-Free and Highly Crystalline NitrateContaining Zn-Al Layered Double Hydroxides. Z. Anorg. Allg. Chem. 640(11):2203-2207.
Sampieri A, Fetter G, Villafuerte-Castrejon ME, Tejeda-Cruz A, Bosch P (2011). Twofold role of calcined hydrotalcites in the degradation of methyl parathion pesticide. Beilstein J. Nanotechnol. 2:99-103.
Touisni N, Charmantray F, Helaine V, Forano C, Hecquet L, Mousty C (2013). Optimized immobilization of Transketolase from *E. coli* in MgAl-Layered Double Hydroxides. Colloids Surf. B Biointerfaces 112:452-459.
Vlada A, Birjega R, Matei A, Luculescu C, Mitu B, Dinescu M, Zavoianu R, Pavel OD (2014). Retention of heavy metals on layered double hydroxides thin films deposited by pulsed laser deposition. Appl. Surf. Sci. 302:99-104.
Yao F, Hu H, Xu S, Huo R, Zhao Z, Zhang F, Xu F (2015). Preparation and Regulating Cell Adhesion of Anion-Exchangeable Layered Double Hydroxide Micropatterned Arrays. ACS Appl. Mater. Interfaces 7(7):3882-3887.
Zhang K, Xu ZP, Lu J, Tang ZY, Zhao HJ, Good DA, Wei MQ (2014). Potential for Layered Double Hydroxides-Based, Innovative Drug Delivery Systems. Int. J. Mol. Sci. 15(5):7409-7428.

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