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Study of the Enzymatic Capacity of *Kluyveromyces marxianus* for the Synthesis of Esters

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Keywords
Biotechnology · Enzymatic background · Esters · *Kluyveromyces marxianus* · Protein

Abstract
Recently, biotechnological opportunities have been found in non-*Saccharomyces* yeasts because they possess metabolic characteristics that lead to the production of compounds of interest. It has been observed that *Kluyveromyces marxianus* has a great potential in the production of esters, which are aromatic compounds of industrial importance. The genetic bases that govern the synthesis of esters include a large group of enzymes, among which the most important are alcohol acetyl transferases (AATases) and esterases (AEATases), and it is known that some are present in *K. marxianus*, because it has genetic characteristics like *S. cerevisiae*. It also has a physiology suitable for biotechnological use since it is the eukaryotic microorganism with the fastest growth rate and has a wide range of thermotolerance with respect to other yeasts. In this work, the enzymatic background of *K. marxianus* involved in the synthesis of esters is analyzed, based on the sequences reported in the NCBI database.

Introduction

Modern industry demands the use of biotechnological processes that allow the production of compounds of commercial interest and can be classified as natural and safe for the human being. In this sense, yeasts play an important role in the production of beverages, foods, and enzymes since their metabolites are considered as natural products [Morrisey et al., 2015]. In this way it has been demonstrated that *Kluyveromyces marxianus* yeast is of biotechnological interest, since it has the capacity to ferment a wide group of compounds such as glucose, lactose, raffinose, sucrose, and inulin, as well as its safe handling status for the human being, since it has the classification GRAS (FDA) [FDA, 2019] and QPS (EFSA) [EFSA, 2019], which makes it suitable for use in the production of food grade compounds [Campos-García et al., 2018; Martynova et al., 2016].

The genus *Kluyveromyces* was described by Van der Walt in 1971, and is a genus comprising six species (*aestuarii*, *dobzhanskii*, *lactis*, *marxianus*, *nonfermentans* and *wickerhamii*) [Löbs et al., 2016]. However, *K. marxianus* has several advantages over the other species not only of its own genus but also with respect to non-*Saccharomyces*

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Group name/abbreviature	Reaction	e.g., products	Authors
Alcohol acetyl transferase/ AATases	Condensation of acetyl-CoA with an alcohol	Ethyl acetate, isoamyl acetate, phenyl ethyl acetate	Plata et al., 2003; Löser et al., 2013; Gethins et al., 2015; Löbs et al., 2016; Fasoli et al., 2015
Esterases/AEATases	Condensation of ethanol with an acyl-CoA	Ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate	Campos-García et al., 2018; Fasoli et al., 2015
Alcohol dehydrogenases/ ADHs	Dehydrogenation of hemiacetals	Methyl formate, ethyl formate, methyl acetate, ethyl acetate	Löbs et al., 2017
Monoacylglycerol lipases/ MGLs	Condensation of ethanol with acyl-CoA	ethyl acetate	Gajewski et al., 2017
Baeyer-Villiger monooxygenases/BVMOs	Dehydrogenation of ketones	Undecyl acetate, dodecyl acetate, methyl acetate	Beier et al., 2014; Alves et al., 2015; Kotani et al., 2003

Fig. 1. Enzymes implicated in microbial esterification.

yeasts. The main advantage consists of being the eukaryotic microorganism with the highest growth rate. This is made evident by the fact that its doubling time is 79.8 min, derived from which it has a high yield of metabolites and biomass [González-Hernandez et al., 2018]. It is thermotolerant since it grows in a range of temperatures from 4 to 52 °C, allowing its use in processes that require high temperatures, which prevents the growth of microorganisms sensitive to heat [Inokuma et al., 2015].

Phylogeny and Physiology of *K. marxianus*

The yeast *K. marxianus* is a strain closely related to *Saccharomyces cerevisiae*, since a syntenic conservation analysis indicated that 49.8% of its genes are syntenic. Likewise, 22.7% of *S. cerevisiae* genes are orthologs of *K. marxianus*, which is why which the latter has a good ability to produce aromas and good adaptation [Lertwattanasakul et al., 2015; Lin et al., 2016]. This strain is also phylogenetically related to *Kluyveromyces lactis* and shares 1,552 genes with it [Kusano et al., 1999], so that both possess the *LAC12* and *LAC4* genes that are responsible for the coding of lactose permease and β-galactosidase, respectively, which allows them to ferment lactose. However, only *K. marxianus* can ferment inulin because it possesses the *INU1* gene, making it useful to grow on plant tissues [Molina et al., 2015]. It has also been shown that this yeast can degrade up to 90% of fructans during bread making [Stribny et al., 2016a].

The yeast *K. marxianus* presents a pyruvate metabolism respiro-fermentative, which indicates that it can generate the energy required by oxidative phosphorylation or by the Krebs cycle. This yeast is also Crabtree negative, which means that its growth can be controlled by the amount of oxygen added to the medium. This is useful during anaerobic fermentation, since it is under these conditions is that ethanol is produced [Hagman et al., 2007; Lodder et al., 1952]. *K. marxianus* stands out as a yeast of biotechnological interest because, like *S. cerevisiae*, it has the faculty to carry out enzymatic esterification through a cellular detoxification process [Zelner et al., 2013]. For this to happen, the energy provided by the thioester bond of an acyl donor is required, of which it is known that the one with the greatest cellular abundance is that which comes from the decarboxylation of pyruvate during the Krebs cycle [Robinson et al., 2014], although it has also been observed that there are other mechanisms of microbial esterification which involve the oxidation of hemiacetals and oxidation of ketones (Fig. 1) [Gamero et al., 2016; Orru et al., 2011; Park et al., 2009].

Enzymes Involved in the Synthesis of Esters

The genetic basis on which the aromatic synthesis in yeasts is based is complex, since the aromas synthesized are inherent to the particular metabolism of each microorganism, although it can be inferred that, due to the homologous nature of the enzymes involved in the process, these enzymes have the same activity even among different genres [Löser et al., 2015a, b]. Thus, the enzymes responsible for microbial esterification are alcohol acetyl transferases (AATases) (EC 2.3.1.84), esterases (AEATases) (EC 3.1.1.3) [Pires et al., 2014; Plata et al., 2003; Struyf et al., 2018; Takahashi et al., 2017] , alcohol dehydroge-

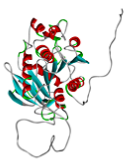
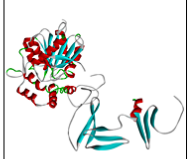
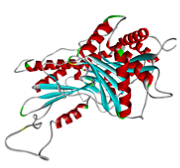
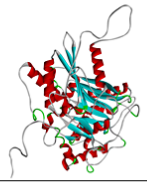
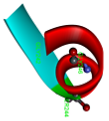
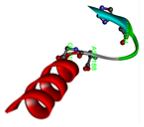
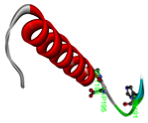
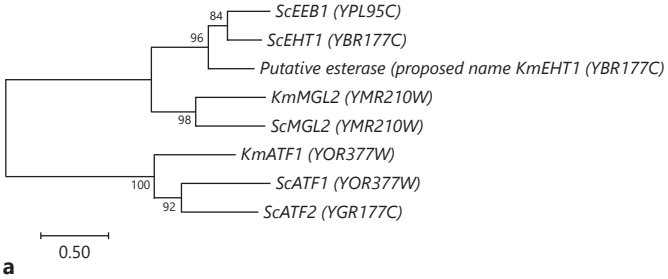
Protein/ systematic name	*KmEht1p/ YBR177C*	*ScEht1p/ YBR177C*	*KmMgl2p/ YMR210W*	*ScMgl2p/ YMR210W*	*ScEeb1p/ YPL095C*	*KmAtf1p/ YOR377W*	*ScATf1p/ YOR377W*
Predicted protein structure							
Predicted structure of active site							
Position of catalytic site/ total length	S244, D391, H420/ 448aa	S247, D395, H423/ 452aa	S223, D355, H383/ 438aa	S232, D364, H392/ 450aa	S251, D399, H428/ 457aa	H184, D188, G189/ 458aa	H191, D194, G195/ 526aa
Position/ conserved sequence	GFSFG	245–249/ GCSFG	221–225/ GVSLG	230–234/ GFSLG	249–253/ GTSFG	188–189/ HVVSDG	191–195/ HCMSDG
Gene localization	Chromosome I	Chromosome II	Chromosome VII	Chromosome XIII	Chromosome XVII	Chromosome III	Chromosome XV

Fig. 2. Three-dimensional protein predictions of esterases based on the reciprocal sequences obtained from the NCBI and modeled using the RaptorX bioinformatics tool.

nases (ADHs; EC 1.1.1.1) [Kusano et al., 1999], monoacylglycerol lipases (MGLs; EC3.1.1.33) [Schneiderbanger et al., 2016; van Rijswick et al., 2019], and Baeyer-Villiger monooxygenases (BVMOs; EC 1.14.13.92) [Ferroni et al., 2016]. However, a new family has recently been discovered that includes the enzyme *Eat1p* of *Wickerhamomyces anomalus* (putative in *S. cerevisiae*) and that has catalytic activity on the synthesis of ethyl acetate [Kruis et al., 2017].

Alcohol Acetyl Transferases

The enzymes that make up the group of the AATases in yeast are: *Atf1p*, *Atf2p*, and *Lg-Atf1p*, the latter being only found in *S. pastorianus*; however, the genes (*ATF2* and *Lg-ATF1*) diverge from the same ancestor (*ATF1*) [Hagman et al., 2014; Saerens et al., 2010; Wilkowska et al., 2015]. The function of these enzymes is to carry out the catalysis of acetate esters by the condensation of alcohol and acetyl-CoA to form an ester. Due to their enzymatic activity, they are classified within clade II of the BAHD family [Molina et al., 2015]. In this sense, the in silico analyses carried out for the comparison of orthologous genes of *ATF1* and *ATF2* of the different species of *Kluyveromyces* with respect to *S. cerevisiae* showed that within the genus *Kluyveromyces* the three species that have been sequenced (*K. lactis*, *K. marxianus*, and *K. dobzhanskii*) contain genes encoding AATases. Likewise, the homology level of *K. marxianus* with respect to *S. cerevisiae* is variable among the proteins encoded by orthologs. However, it is known that the most important domain is usually located in the center of the protein and is formed by the motif activation domain HxxxDG, where x can be substituted for any amino acid [Mason and Dufour, 2000; Selvaraju et al., 2016]. The importance of this domain is that it is the catalytic site of the enzyme and its mechanism of action causes the histidine residue to remove a proton from the oxygen or nitrogen of the acceptor group, so that later the nucleophilic attack on the carbonyl carbon takes place at the thioester bond of the acyl donor [Carrasco Orellana et al., 2018]. Protein predictions on *ScATF1p* and *KmATF1p* indicated that this domain is found at amino acids 191–196 and 184–189 of *S. cerevisiae* and *K. marxianus*, respectively (Fig. 2). On the



a

Gene	Product	Accession No.
ScEEB1	*ScEeb1p*	EEU09158
ScEHT1	*ScEht1p*	AJP84079
ScMGL2	*ScMgl2p*	NP_013937
ScEAT1	*ScEat1p*	EDZ72113
ScATF1	*ScAtf1p*	EGA72844
ScATF2	*ScAtf2p*	ONH72747
KmEHT1	*KmAht1p*	XP_022674303
KmMGL2	*KmMgl2p*	BAP73297
KmEAT1	*KmEat1p*	BAP71564
KmATF1	*KmAtf1p*	XP_022675344

b

	Similarity/identity, %					
	ScEeb1p	*ScEht1p*	*ScMgl2p*	*ScEat1p***	*ScAtf1p*	*ScAtf2*
*KmEht1p****	97–50	97–55	–	–	–	–
*KmMgl2p****	94–30	91–31	96–45	–	–	–
*KmEat1p**	–	–	–	94-42	–	–
KmAtf1p	–	–	–	–	92–37	95–33

c

Fig. 3. Relationships and homology of the homologous genes of *K. marxianus and S. cerevisiae*. **a** Maximum likelihood phylogenetic tree based on the JTT model, using 1,000 bootstrap samples as a validation method. **b** Sequences of AATeases/AEATases present in *S. cerevisiae* and *K. marxianus*. **c** Relationships by homology and identity of the AATases and AEATases. Identities with a higher percentage appear on gray background. * Uncharacterized-putative protein Eat1p/YGR015C; ** putative protein *Eat1p*/YGR015C; *** putative esterase *Mgl2p*/YMR210W; **** α/β hydrolase 2-proposed *Eht1*/YBR177C.

other hand, the second important domain that characterizes the enzymes of the BADH family consists of the sequence DFGWG [Menendez-Bravo et al., 2017; Moglia et al., 2016], which is not found in the sequences mentioned above (data not shown). Gethins et al. [2015] conducted synteny studies and observed that the *KmATF2* gene was an ortholog of *ScATF2*. However, the in silico analyses carried out by means of the reciprocal comparison between the protein sequences of *ScATF1* and the sequence of *KmATF* found in the database of the NCBI indicated an identity level of 37%, while the same analysis between the *ScATF2* and *KmATF* sequences resulted in a 33% identity, which could suggest that in fact the *ScATF1* gene is in fact the ancestral ortholog of the *KmATF* gene. This fact it can also be observed in the proximity between the branches shown in the phylogenetic tree (Fig. 3a, c).

Esterases

The synthesis of ethyl esters in yeast is produced by the condensation of medium chain fatty acids (C6–C14) with ethanol [Kruis et al., 2017; Zhuang et al., 2015]. This reaction requires the catalytic action of esterases, which are proteins with dual nature, since they can act as ethanol *O*-acyltransferases and as thioesterases (*Eht1p*, *Eeb1p*) [Ichikawa et al., 1991; Yin et al., 2019; Zelner et al., 2013]. However, *Mgl2p* is a protein that also possesses lipase activity and therefore is responsible for maintaining cellular lipid balance [Gajewski et al., 2017; Manda et al., 2017; Robinson et al., 2014]. The function of the AEATases is to obtain the CoA of medium chain fatty acid esters by hydrolyzing them, and to a lesser degree participating in their formation by transesterification. In this sense, the preference of the enzymes on the substrates is variable, since *Eht1p* has a preference for octanoyl-CoA (C8) and to a lesser degree alcohols up to C14, this has been attributed to the geometry of the substrate within the catalytic site of the *α/β* hydrolase sheet [Knight et al., 2014; Procopio et al., 2011]. The BLASTP analyses carried out on the AEATases showed that *K. marxi*anus only encodes *KmEht1p* and *KmMgl2p*, and the homology is 55 and 45%, respectively, with respect to *ScEht1p* and *ScMgl2p*, being the last putative (Fig. 3). However, due to its orthologous nature, it can be deduced that *K. marxianus* catalyzes the transesterification of fatty acid esters [Stribny et al., 2016b]. For this reaction, the active site is composed of a Ser-Asp-His catalytic triad [Robinson et al., 2014] (Fig. 4)

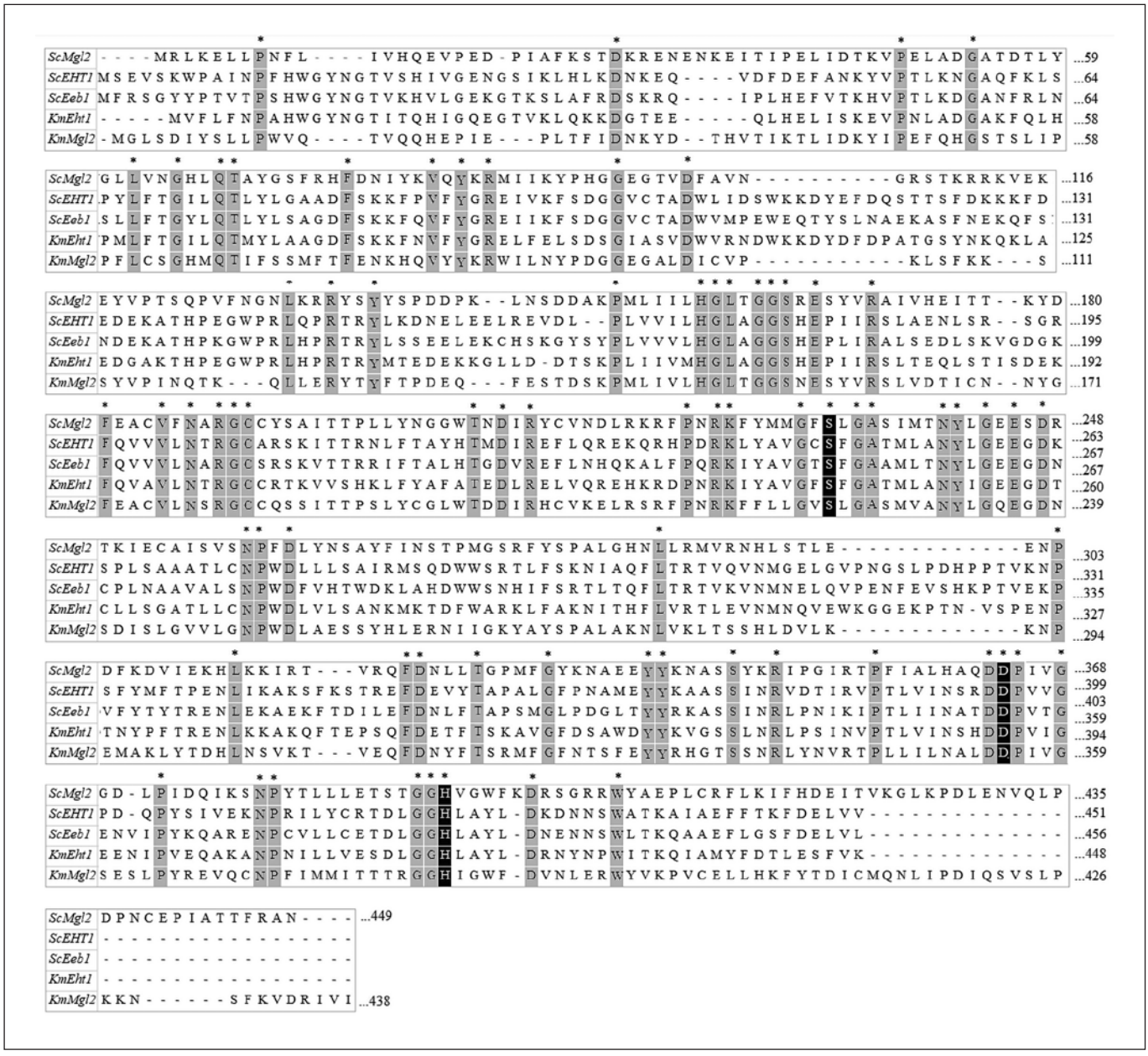


Fig. 4. Alignment of peptide sequences of proteins: putative Mgl2p (NP-013937), Eht1p (AJP84079), and Eeb1p (EEU09158), from S. cerevisiae and their homolog Mgl2p (BAP73297). Eht1p (XP_022674303) from *K. marxianus*, of gray background, appear highly conserved sites (100%). The amino acids that are believed to make up the catalytic site have been highlighted with a black background. Created with MEGA7.0 software through ClustalW.

that is usually located around the α/β hydrolase folding. These proteins also usually possess the GxSxG consensus sequence [Bornscheuer, 2002] (Fig. 2). Likewise, the analyses carried out on the protein sequences indicated that for *KmEht1p*, the catalytic site is found in amino acids S223, D355, and H383 and, similar to *ScEht1p*, the consensus sequence GxSxG in *ScEHT1p* (S247, D395, H423). The remaining proteins and three-dimensional conformation showed a loop corresponding to the folded sheet characteristic of the family of the α/β hydrolases which the amino acids that make up the catalytic site surround. The mechanism of action consists first of the union between

the enzyme and the substrate that is stabilized by the serine residue, that is stabilized by the adjacent amino acids of histidine or asparagine. Once the alcohol release occurs the enzyme binds to the acil-CoA, so it predisposes for the nucleophilic attack to occur and the ester is formed plus free CoA [Bornscheuer, 2002]. Studies conducted on the catalytic core showed that the site-directed mutation in the S and H residues does not affect the protein structure [Knight et al., 2014; Kruis et al., 2017]. The consensus sequence (GxSxG) that is shared between the lipases and esterases, and that usually accompanies the serine residue of the hydrolases, was found in the proteins analyzed, being located in residues 242 (GCSFG) and 245 (GFSFG) for *ScEht1p* and *KmEHT1p*, respectively [Glaeid Ghram et al., 2016]. The conserved sites of the protein encoded by *MGL2* can be seen in Figure 2. With respect to this gene, it has been observed that its product (*ScMgl2p*) exhibits a HxxxD motif (HAQDD) at the terminal carbon. However, this domain is not present in *KmMgl2p*. It is also thought that *MGL2* serves as a genetic resource for yeast since *Mgl2p* is usually more active when other genes (ATFs) are repressed [Löser et al., 2013].

Alcohol Dehydrogenases

ADHs are enzymes by which esters are synthesized from alcohols and aldehydes that are toxic to the cell [Borrull et al., 2015; Keller et al., 2017]. In addition, oxidation of the hemiacetal makes it possible to reduce NAD+ and thereby generate energy (Orru et al., 2011). This type of esterification is carried out from primary and secondary alcohols, as well as aldehydes and ketones [Selvaraju et al., 2016]. In *S. cerevisiae* it has been shown that *Adh1p* and *Adh2p* catalyze the reaction of ethyl acetate. However, recent studies on the ADHs of *K. marxianus* show that the only ADH that seems to have an active role in the production of ethyl acetate is *Adh7p* (BAO40648), a zinc-dependent enzyme with preference for NADP(H), and whose homology with *Adhp* of *S. cerevisiae* is low [Llorente et al., 2000]. However, it has a high level of homology with the ADHs of certain bacteria (*Snodgrassella alvi* [76%], *Acinetobacter towneri* [74%]) [Lin et al., 2016; Llorente et al., 2000].

Baeyer-Villiger Monooxygenases

BVMOs are enzymes dependent on FAD or NAD that catalyze the degradation of aliphatic ketones for the formation of branched esters, which are hydrolyzed to yield an acid and an alcohol [Beier et al., 2014; Kim et al., 2018]. By means of this mechanism, compounds of industrial interest such as dodecyl acetate (odor of wax) can be obtained. Although at first it was believed that this was exclusive of bacteria, it has recently been found to also occur in some yeasts [Alves et al., 2015]. On the other hand, the analyses carried out on *K. marxianus* by BLASTP (nr) on the sequences reported by Beier [2017], corresponding to *C. albicans* (XP_720980), indicated a homology with the monooxygenase (BAP69657) of *K. marxianus* of 26 and 22%. However, the protein of *K. marxianus* only possesses the folding of Rossmann 2 (GxGxxG/A), located in the amino acid 191 (GNGSSA), indicative of its preference for the NAD, although the sequences (FxGxxxW [P/D], [A/G]GxWxxxx[F/Y]P[GMxxxD) are not present. On the other hand, the newly discovered sequence (Dx[I/L][V/I]xxTG[Y/F]) is located at position 257 (DYIIWATGF), which places it as BVMO I, so it can be assumed that this enzyme plays an active role during the formation of some esters; where it does not it has been possible to establish the dominant residues for the preference of the substrate [Mylona et al., 2016].

Conclusion

The increase in the demand of isolated aromas from natural sources makes it necessary to acquire the knowledge to produce these compounds through technological processes that lead to obtaining safe products to consume and with the qualities required by the final consumer. Knowledge in the genetic and metabolic analysis of the mechanism of the genes involved in the formation of esters implies a biotechnological improvement to increase the production of these compounds [Kruis et al., 2019].

This study shows that it is possible that the capacity of ester synthesis in *K. marxianus* is corrected in the presence of the *EHT1, EAT1, MGL2, ATF1*, and *ADH7* genes, which encode the production of enzymes involved in the production of esters. As a saber, the *ATF1* gene participates in the production of acetate esters, being the ortholog of the *ATF1* gene of *S. cerevisiae* with which it shares a level of homology of 37%. But it has been shown that it has the same catalytic site, which is the most important domain and where all the activity takes place for the formation of these aromas. As a result, enzymes encoded by this gene have been created and are actively involved in the production of ethyl acetate and isoamyl acetate in this yeast [Manda et al., 2017; Naresh Kumar et al., 2018]. On

the other hand, the enzymes encoded by the *EHT1* and *EAT1* genes participate in the synthesis of aromas such as ethyl ethanoate, ethyl hexanoate, and ethyl decanoate, among others [Celińska et al., 2018; Löbs et al., 2017]. Although these last two genes have been found in the putative manner, this study has also found metabolic activity has also occurred in the yeast genome, and it has been said that it is very likely that these genes have stopped being putative in some later studies. On the other hand, the *MGL2* gene also encodes a protein involved in the synthesis of ethyl esters, like the two previous genes, with the difference that it has lipase activity. This is an important activity as it is important that the proteins encoded by the same gender regulate the cellular detoxification of yeast. Regarding the *ADH7* gene, it has been shown to be an important gene for the synthesis of ethyl acetate [Llorente et al., 2000]. This shows that the synthesis of esters is not only due to the presence of AATases and AEATases, but also that the metabolic mechanisms are linked to protect the integrity of the cell before the possible loss of functionality of one of the mechanisms of survival. With this bibliographic study, it is clear that not all enzymes involved in the formation of esters have yet been discovered, since the functionality of other enzymes such as BVMOS has not yet been tested; however, it is likely that they are involved in the formation of esters in some way.

Statement of Ethics

No ethical approval was required since an in vivo investigation with living beings was not conducted, and therefore this document was not submitted for authorization by an internal review committee.

Disclosure Statement

The authors declare that there is no conflict of interest regarding the publication of this article.

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Author Contributions

All authors contributed to the elaboration of this document. The contributions according to the ICMJE authorship criteria are as follows: Francisco Javier Reyes-Sánchez, Jesús Bernardo Páez-Lerma, and Juan Antonio Rojas-Contreras: substantial contributions to the conception or design of the work, and the acquisition, analysis, or interpretation of data for the work. Javier López-Miranda, Nicolás Óscar Soto-Cruz, and Manuel Reinhart-Kirchmayr: drafting the work or revising it critically for important intellectual content. All authors gave final approval of the version to be published.

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