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1 **Uses and advantages of CRISPR/Cas genetic edition in yeasts**

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14 **Abstract:** This review summarizes the use of CRISPR system in yeasts, identifying advantages and
15 disadvantages of its applications. 39 articles were evaluated including 12 articles that discussed the
16 advantages of new CRISPR systems that improved the initial system, and another 27 were
17 evaluated, among these: three were applications in *Cryptococcus neoformans*, four in *candida* sp., three
18 in *Schizosaccharomyces pombe*, nine in *Saccharomyces cerevisiae*, four in *Yarrowia lipolytica*, and four in
19 industrially important yeasts such as *Pichia pastoris* and *Saccharomyces pastorianus*. It was concluded
20 that the CRISPR system is one of the most versatile genetic editing systems available nowadays. It
21 can be applied in different organisms for several effects including gene knock-outs, performing
22 point mutations, gene expression, or even applying multiple edition operations in several genes.
23 However, we recognize that numerous studies lack a control group of the mutated strains, which
24 leaves many questions unanswered. For instance, the extent and precision of this techniques, it also
25 represents a risk to biosecurity standards. Therefore, this review shows the compilation of CRISPR
26 system information, which could be used to generate different alternatives in the industry and
27 clinical fields

28 **Keywords:** CRISPR System, Yeast, gene edition

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1. Introduction

In the past, invasive fungal infections were rare enough to be thought of as unimportant. However, yeasts are able to colonize different environments rich in carbon compounds [1]. The study of pathogenic fungi is of great importance due to the increase of clinical cases and the few available antifungals. Additionally, there are weaknesses in the spectrum, potency, and pharmacokinetics of many therapeutic compounds. Research on the study of these pathogens focuses on their pathogenicity, virulence, and differentiation of fungal cells from mammalian host cells, due to their great similarity [2]. Yeasts are also known for their industrial applications which include food production and drug development. The genus with greater industrial use is Saccharomyces, it is also a frequent model for biotechnological studies [3].

The CRISPR system (clustered regularly interspaced short palindromic repeats) was identified as a defense mechanism against exogenous DNA molecules that could affect and alter the cell's DNA, such as phage or plasmid infections and it's thought of as an adaptive immunity system in prokaryotes. [1]. Over the years, many studies were conducted to understand the function of the system in prokaryotes and the structures of the molecules involved in this system. It was not until 2012 that Jennifer Doudna a Canadian researcher and Emmanuelle Charpentier a French researcher reinvented the CRISPR system and repurposed it as a tool for genetic engineering [2]. This system has brought a revolution upon contemporary biology in the modification of genetic information.

The CRISPR-CAS system is divided into 2 classes; those of class 1 are those that need a large complex of proteins that carry out the action of DNA degradation through a sequence of guide RNA. Those of class 2 are systems that need a single effector endonuclease protein guided by an RNA to carry out the neutralization of the invasive genome. This is the case of the CRISPR-Cas9 and CRISPR-Cpf1 system [3]. Cas9 endonuclease is the most used in genetic editing for Eukaryotic microorganisms such as yeasts. The class 2 system is composed of an endonuclease (Cas9 or Cpf1), a short sequence of RNA and the sequence PAMs short DNA sequences (from 3 to 5 bp) called protospacer adjacent motifs [4]. The endonuclease has two lobes: a) recognition system (REC), which is divided into two domains REC1 and REC2, and b) nuclease activity system (NUC), it has the RuvC, HNH and PI domains recognized by the PAM sequence in the DNA sequence [5]. This endonuclease protein performs its activity using the RuvC and HNH domains three nucleotides upstream of the PAM sequence. The guide RNA (sgRNA) consists of a chimeric RNA that retains two fundamental characteristics: a 5 'sequence that determines the DNA target site by pairing Watson-Crick bases (crRNA) and a 3' duplex RNA structure that binds to Cas9 (tracrRNA) [6]. In this review, we describe the application of this system in two species of yeasts of clinical importance, *Candida sp*, and *Cryptococcus neoformans*, as well as industrially important species *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, and *Schizosaccharomyces pombe*.

2. Study of editing of genomes by CRISPR-Cas in yeasts of industrial and clinical importance.

Yeasts of clinical importance

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83 Candida Sp is a genus of yeasts and is the most common cause of fungal infections worldwide; This
84 opportunistic infection has a global incidence of around 700,000 cases per year [4]. Although the
85 genus has around 20 human pathogenic species, the CRISPR-Cas9 system to date has been used in
86 only three species of them: *C. albicans*, *C. glabrata*, and *C. parapsilosis*.

87 The CRISPR-Cas9 system was used to modify the genes ADE2, MET15 and SOK2 in *C. glabrata*, which
88 were located in different chromosomes. The study compared with the application of a SAT1 cassette,
89 a more stablished method of genetic editing. They demonstrated that the CRISPR system is three
90 times more efficient compared to the alternative method of genome editing [5]. The efficiency of
91 CRISPR-Cas9 was not homogenous between all target genes, it was higher for MET15 compared to
92 ADE2 for example. In addition, it is known that efficiency varies in the same target gene depending
93 on the sgRNA sequence used [6].

94 In other study, the technique was applied to generate knock-out mutations in two genes of *C. glabrata*
95 which were thought to be associated with the infection process. After successfully performing the
96 desired mutations with a CRISPR-Cas9 system, the mutants were evaluated in the animal infection
97 model *D. melanogaster*. Their results show the mutants were less virulent than the WT strains,
98 concluding that these two genes must participate in the infection process of *C. glabrata* in vivo.[7].

99 The development of modifications in the CRISPR-Cas9 system is frequent. These modifications
100 depend to a great extent on the known genotypic characteristics of the microorganism. In *C.*
101 *parapsilosis* an editing system was developed that consisted of a single step of transformation,
102 expressing the CAS9 gene only when the plasmid was present. in addition, this modification
103 allowed to easily eliminate the transformed strains. It is the first system applied in C. parapsilosis
104 using a marker of resistance to nourseothricin in which they published the genes (URA3 and
105 ADE2) by consecutive transformation with two plasmids expressing different sgRNAs to increase
106 the efficiency of gene editing. This gene editing/deletion system could be easily used and could
107 be applied to generate a large number of genetic knock-outs [8].

108 *Cryptococcus neoformans:* Cryptococcosis is an opportunistic fungal infection, acquired by the
109 inhalation of fungal propagules present in the environment; is a potentially fatal infection that affects
110 the lungs and the central nervous system (CNS) in immunosuppressed and immunocompetent
111 individuals [9]. The infection is caused by two species, namely: 1) *Cryptococcus neoformans* var. *grubii*
112 (serotype A), var. *neoformans* (serotype D) and a hybrid corresponding to serotype AD and 2)
113 *Cryptococcus gattii* (serotypes B and C). These species present phenotypic, genotypic and
114 epidemiological differences, as well as in their geographic distribution, in addition, it is a mycosis
115 that presents tropism by the CNS causing meningitis, different virulence factors have been described,
116 implicated in pathogenicity as the capsular size and the production of melanin [10]. Therefore, several
117 investigations have focused on the establishment of the CRISPR-Cas9 system to carry out the selective
118 elimination of virulence and pathogenicity genes in Cryptococcus neoformans.

119 This yeast has a low frequency of homologous recombination, especially for strains of serotype D

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120 [11], which has hindered molecular genetic studies in the past. In 2016 Arras S., and collaborators
121 evaluated the use of CRISPR Class 2 for the study of pathogenicity in *Cryptococcus neoformans.*
122 Initially, they expressed a derivative of *Streptococcus pyogenes* nuclease Cas9 in *C. neoformans* and
123 showed that it has no effect on growth, in addition, they evaluated the production of virulence factors
124 in a murine model. They tested CAS9 in combination with multiple self-cleaving guide RNAs
125 targeting the *ADE2* gene encoding phosphoribosilaminoamidazole carboxylase. This revealed that
126 CRISPR functionality in *C. neoformans* depends on the CAS9 construct being stably integrated into
127 the genome, whereas the transient expression of the guide RNA is enough to increase the rates of
128 homologous recombination. This highlights the versatility of this genetic system. Furthermore, the
129 presence of CRISPR nuclease does not influence virulence in a murine model, they successfully
130 demonstrated that this system is compatible with pathogenicity studies in *C. neoformans* [12].

131 When the CRISPR-Cas9 system persists in the host cells, cytotoxicity effects may occur, which could
132 block the performed genetic manipulation. This system is a powerful method to perform directed
133 mutagenesis in organisms that present low recombination frequencies and for functional genomic
134 studies. Wang Y., and collaborators reported a method to spontaneously eliminate the CRISPR-Cas9
135 system without affecting its robust editing function. They expressed the unique guiding RNA under
136 the driver of an endogenous U6 promoter and the Cas9 endonuclease optimized in human codon
137 with an ACT1 promoter. This system efficiently generated gene alteration through homology-
138 directed repair by electroporation in yeasts, spontaneous elimination of the system was demonstrated
139 through a CRISPR-Cas9 expression cis arrangement, allowing the validation of genetic functions
140 through subsequent complementation and has the potential to minimize the effects outside the target
141 [13].

142 The biobalistic transformation in C. neoformans, is a tool used for editing the genome where the
143 introduced DNA is inherited in a stable manner, the transformation efficiency and the homologous
144 integration rate is low (approximately 1-10%). The development of Transient CRISPR -Cas9 together
145 with the electroporation system (TRACE) proved useful to increase the rate of transformation since
146 it efficiently integrated new material into the genome due to double-strand breaks created in specific
147 sites by the CRISPR-Cas9 system and the high transformation efficiency of electroporation. This
148 system can effectively eliminate multiple genes in a single transformation, as well as insert DNAs
149 into a designated genetic site without any homologous sequence, which opens many other
150 applications [14]. CRISPR can be used as a tool for the interruption of genes of high efficiency in
151 *C. neoformans*, it is a useful system to understand this organism and its pathogenicity.

152 **Yeasts of industrial importance in biotechnological processes**

153 *Saccharomyces cerevisiae:* It is the most used yeast in industrial processes, followed by *S. bayanus* and
154 *S. pastorianus*. In addition, it is the best studied yeast both in its physiological and genetic
155 characteristics, it was even the first Eukaryotic cell to be sequenced [15]. In the industry editing of its
156 genome has been implemented in the past to optimize processes among other applications. Studies
157 have been conducted using the CRISPR-Cas9 technology to reduce production of ethyl carbamate
158 (EC), a potential carcinogen which is formed during the process of fermentation of ethanol in this

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159 yeast. It has also been used to perform a "knock out" of the CAR1 gene involved in the synthesis of
160 the EC. Process, leaving no antibiotic marker genes or any residual sequence in the vicinity of the
161 CAR1 gene. To inactivate the gene, they introduced a nonsense mutation in the start codon of glycine
162 (Gln) transforming it into a stop codon (TAA) by homologous recombination and designed another
163 experiment that performed the elimination of the traditional NHEJ, eliminating 1002 bp of the gene.
164 To achieve their goal they used two plasmids, one containing the CRISPR system and the other
165 containing the sgRNA. They demonstrated that CRISPR-Cas9-mediated inactivation of the CAR1
166 gene led to a significant reduction in the specific activity of arginase, urea and CD. Furthermore,
167 when comparing the phenotype of the mutants with the wild-type strains, they did not observe
168 statistically significant differences in the growth of the yeast in the culture, nor in the percentage of
169 glucose consumption and ethanol production, which shows that the mutation did not affect the
170 industrial yield of the production, and on the contrary, it reduced the levels of CE by 60% in culture
171 [16].
172
173 In recent years, new CRISPR designs have been generated in *S. cerevisiae*. Dashko S and collaborators
174 in 2018, implemented a new family of programmable endonucleases by CRISPR-Cas class II. Using
175 the constitutively expressed endonuclease Cpf1 of *Francisella novicida* (Fn Cpf1), RNA cleavage of
176 DNA guided by specific genomic loci can mediate. When evaluating this nuclease, they showed that
177 it is not toxic to the yeast and does not interfere with the growth in the culture medium. They
178 determined that the PAM sequence that recognizes this endonuclease is TTN and that Fn Cpf1 is able
179 to perform a directed edition guided by a crRNA and in turn perform point mutations in the ADE2
180 gene by inserting a stop codon. Fn Cpf1 showed an efficiency of up to 100% for the repair of DNA
181 recombination [17].

182 A novel method for the editing of the genome by CRISPR-Cas9 in S. cerevisiae, consists of introducing
183 a cut-off site in a specific genomic location, followed by the integration of a sequence edited in the
184 same location in a way without scars. They published sequences of the promoter GAL1 and GAL80,
185 they managed to over-express both genes in the yeast, and they showed that the production of agrosa
186 was proportional to the fluorescence emitted by the marker, to achieve this method they had to insert
187 the PAM sequence in the region in a synthetic way. close to the promoter to achieve that the CRISPR
188 system will identify the site in which it should be inserted to stimulate the promoter and overextend
189 the gene [18]. Protocols have also been developed that produce integrations without scars and
190 without DNA markers using a 20 bp sgRNA immediately following the PAM (NGG) sequence [19].

191 The CRISPR-Cas9 multiplex system was used for the genome engineering of up to 5 different
192 genomic loci in a single transformation step in the yeast, with this methodology. Jakočinas T et al; in
193 2015, managed to overexpress 41 times the mevalonate, an important intermediate metabolite in
194 cholesterol biosynthesis in mutated strains, which makes this methodology a useful tool to increase
195 the production of an industrial molecule[20]. In addition, libraries of high efficiency integration
196 plasmids have been generated to be implemented in the CRISPR-Cas9 system. The DOE Joint
197 BioEnergy Institute, Emeryville of California used a set of cloning-free tools that allows rapid and
198 easy genetic modification of strains in *S. cerevisiae*, using different plasmids, which presented an

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199 efficiency above 95% in 23 genomic loci characterized. The toolkit described in this paper provides a
200 rapid approach to examine multiple gene expression contexts simultaneously [21].

201 In Denmark they developed kit with EasyClone-MarkerFree vectors using the CRISPR / Cas9 system
202 that facilitates the integration of linearized expression cassettes at defined genomic loci, expressing
203 the (sgRNA) from a set of RNAG helper vectors. Using that set of genomic engineering vectors,
204 simple inserts are obtained with 90-100% and triple with 60-70% focusing efficiency. The EasyClone-
205 MarkerFree vector toolkit can be used to simultaneously enter one to three integration cassettes into
206 the genome of *S. cerevisiae*, without the use of selection markers. Integration cassettes can be
207 constructed for overexpression of one or two genes per integration site; In that study, they
208 successfully integrated up to six genes in a single transformation with a targeting efficiency of 60 to
209 70%[22].

210 Using the CRISPR-Cas9 system, they integrated by homologous recombination (HR) the XYL1, XYL2
211 and XYL3 genes in the loci PHO13 and ALD6 (involved in the production of acetate), to achieve the
212 overexpression of heterologous genes and the cancellation of endogenous genes simultaneously in
213 strains of *S. cerevisiae*. For the construction of the mutated strain, they used the sgRNA directed to
214 PHO13 and ALD6 sequentially to replace the genes; in the study they suggest that the sequential
215 integration process can be shortened by a transformation with a plasmid carrying both sgRNA
216 together using multiplex methods. All of the above was carried out in order to generate at an
217 industrial level strains that have higher levels of fermentation and that do not represent a public
218 health hazard, since they do not present resistance genes inserted in the mutation process [23].

219 *S. cerevisiae*, has been used in biotechnological processes, an example of this is the evaluation to the
220 resistance to an antiparasitic against the malaria of the family spiroindolonas, the disadvantage with
221 this medicine is that it becomes less active by mutations in an ATPase type P of the parasite. To verify
222 this pattern in the parasite and that the mutations in the P-type ATPase enzyme confers resistance,
223 they used the cellular model of S. cerevisiae, mutated (using CRISPR) the gene that encodes a P-type
224 ATPase (ScPMA1 and ScYRR1) and exposed the cells mutated to spiroindolones (KAE609) to see if
225 they acquired the resistance. These experiments confirmed that mutations in ScPMA1 and ScYRR1
226 cause a 2.5 fold increase in resistance to KAE609, however, ScYRR1 does not appear to be the main
227 target of KAE609. On the other hand it was shown that the ScPMA1 gene mutation is the target
228 molecule of a KAE609 and its mutation is directly related to the resistance [24].

229 CRISPR in *S. cerevisiae*, is a very useful system at an industrial level, it has been implemented for the
230 editing of the genome with different approaches, the expression of genes that produce a harmful
231 molecule have been inhibited, this technique was also used in the overexpression of genes and in
232 bioengineering. S. cerevisiae, whose genome and functionality of its genes are well known, is a
233 cellular model, very useful for the design of new CRISPR variant methodologies that allow multiplex
234 mutation and open the possibility of generating more profitable strains for the industry in short time
235 and in a very simple way.

236 *Yarrowia lipolytica:* This yeast has become an interesting model due to its ability to store large

237 concentrations of lipids in its interior and its ability to secrete proteins to produce valuable
238 biochemical products at an industrial level [25]. The work in this yeast has focused on its
239 physiological, metabolic and genomic characteristics, specifically in the secretion of proteins, for the
240 use of hydrophobic substrates and the biogenesis of the peroxisome, in addition, of the study of
241 molecules involved in dimorphism, in understanding the Mitochondrial complexity, the biogenesis
242 of the lipid body and lipid homeostasis, advances in the study of molecular biology have been made
243 by explaining the splicing of introns and the alternative splice, among others[26].

244 Being a microorganism completely sequenced and with great industrial advantages, studies have
245 been carried out in which the CRISPR gene editing technique has been implemented to mutate
246 specific genes that improve the production of certain compounds in this yeast. In China, they
247 implemented the CRISPR system to edit the genome with a single plasmid (pCAS1yl or pCAS2yl) by
248 homologous recombination (RH) and non-homologous recombination (NHEJ) for the TRP1 and
249 PEX10 genes [27]. They demonstrated that the highest percentage of efficiency of the system occurred
250 on day 4 of growth, they achieved a simultaneous double and triple gene editing with the plasmid
251 pCAS1yl by NHEJ. The system with the plasmid pCASyl was successful in different strains of Y.
252 Lipolytica, therefore, this system was more efficient than traditional methods of genome editing and
253 facilitated synthetic biology, metabolic engineering and functional genomic studies.

254 Zhang J Lai and Col in 2018, designed a new CRISPR system called CRISPRi multiplex system for the
255 repression of multiple genes in a single step, in *Y. Lipolytica*, using the Golden-brick assembly method
256 that can assemble different parts without the Initial PCR procedure, which prevents the introduction
257 of new errors in the PCR amplification process and only needs two restriction enzyme sites so that
258 all parts can be assembled in one step [28]. For the design of the CRISPRi system four repressors were
259 used: Cpf1 deactivated with DNase (dCpf1) from *Francisella novicida*, Cas9 deactivated (dCas9) from
260 *Streptococcus pyogenes* and two fusion proteins (dCpf1-KRAB and dCas9-KRAB); In addition, ten
261 gRNAs that were linked to different regions of the GFP gene (green fluorescent protein) were
262 designed and the results indicated that there was no clear correlation between the efficiency of
263 repression and the target sites, regardless of which repressor protein was used. In order to rapidly
264 produce strong gene repression, a multiplex sgRNA strategy was developed in which a high
265 repression efficiency of 85% (dCpf1) and 92% (dCas9) was achieved in a short time by making three
266 different gRNAs towards the GFP gene simultaneously.

267 In this same study, they repressed plural genes vioA, vioB and saw simultaneously and the gene saw
268 using the CRISPRi multiplex system in *Y. lipolytica*. To test the effectiveness of the system, they
269 constructed a VioABE strain containing the protodeoxy-violaceinic acid route (PVA, a pigment
270 derived from tryptophan and its content can be quantified by absorbance) using the dCpf1-Multi and
271 dCas9-Multi vectors, with simple sgRNA and with sgRNA multiplex (3 sgRNA). The transformation
272 was evaluated by PVA in which it was evidenced that when repressing only vioE, the absorbance
273 was reduced to 60% and 40% with the protein dCpf1 and the protein dCas9 respectively, the CRISPRi
274 multiplex system was feasible to implement the repression of multiple genes.

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275 Several protocols have been generated for the genetic editing of *Y. lipolytica*. In 2016b Jassop et al.,
276 Created a new genetic tool Easy-Clone YALI, which allows the construction of genetically modified
277 strains with high efficiency in a simplified way in *Y. lipolytica* through the CRISPR / Cas9 technology.
278 Modulates gene expression with the integration of cassettes in intergenic sites IntC_2, IntC_3, IntD_1,
279 IntE_1 and IntE_3, in addition, did not affect the growth of yeast [29].

280 In yeast *Y. Lipolytica*, the CRISPR-Cas9 system was used to efficiently perform genome alteration, in
281 most articles it was used as LEU2 selection marker, and the PCR technique was used to confirm the
282 alteration of the genome. ; The transformation protocols in the four articles were very similar to each
283 other and are a very useful tool to evaluate the efficiency of these protocols in other yeast species.

284 *Schizosaccharomyces pombe*: It is a yeast highly studied at an industrial and clinical level together with
285 S. cerevisiae, which is widely used at an industrial level for the fermentation of alcoholic beverages
286 such as rum, tequila, and artisan beverages such as Cachaça in Brazil [31]. It is also a yeast used in
287 the wine industry due to its ability to use malic acid and thus reduce the acidity of wine [32]. In
288 addition, it is a fully sequenced microorganism which allows biotechnological studies.
289

290 Jacobs JZ y cola in 2018 at the State University of New Jersey developed a CRISPR-Cas9 system that
291 allowed the genome editing in *S. pombe*. They published the ade6 gene, being mutated causes the
292 accumulation of a red precursor in media with low adenine content to be able to identify the
293 transformed cells, concluded that the CRISPR-Cas9 mutagenesis achieves an almost complete
294 efficiency and eliminates the need for selectable markers. The only vector that expresses Cas9 and
295 sgRNA is marked with URA4, which allows the elimination of the plasmid by selection with 5-
296 fluoroorotic acid and allows the subsequent mutagenesis of additional targets. The built-in rrk1 /
297 Hammerhead Ribozyme cassette, expressed in Pol II, is useful in other situations where RNA
298 expression and defined arbitrary sequences, such as siRNA or lincRNA, is needed and represents an
299 advantage over Pol III RNA systems. The methods and reagents presented here are useful for
300 genomic research in *S. pombe* by allowing rapid and specific genome editing[33].

301 In 2018, they described the CRISPR-Cas9 system to rapidly introduce deletions in the DNA regions
302 that serve as auxotrophic markers in *S. pombe*, these were: leu1-D0, his3-D0 and lys9-D0, ura4-D18.
303 This system consists of a Cas9 gRNA expression vector and a pair of donor DNA plasmids for each
304 deletion. In addition, they reorganized the essential components in the commonly used pREP
305 plasmid series and assembled the corresponding auxotrophic marker gene in these plasmids[34].

306 The developed a cloning-free procedure that uses DNA repair in fission *S. pombe* cells to assemble
307 two linear DNA fragments was performed in 2018 in Beijing, used a circular plasmid encoding Cas9
308 and an amplified sgRNA insert by PCR, both fragments contain only a portion of the URA4 or bsdMX
309 marker, so that only the properly assembled plasmid can confer prototrophy (ability to grow on
310 minimal medium) of uracil or resistance to blasticidin in transformed yeast [35]. In this study they
311 showed that CRISPR-Cas9 based on repair and cloning free allows rapid and efficient point mutation,
312 endogenous N-terminal labeling and elimination of the genomic sequence in fission yeast.

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313 In the yeast *Schizosaccharomyces pombe* in recent years have been developed multiple studies that
314 design various protocols and different mutation alternatives through the CRISPR / Cas9 system that
315 facilitate the mutation of the genome and improve its efficiency to develop genomic studies that
316 explain the function of different genes and at the same time can be used for other cellular models.

317 **CRISPR-Cas system in atypical yeasts.**

318 At present there are described, approximately 900 yeast species, the investigations are focused on a
319 limited number of these species, we wanted to collect some studies on yeasts that are rare as is the
320 case of *Pichia pastoris* and *Saccharomyces pastorianus*, specifically in the genomic edition by CRISPR-
321 Cas, we reflect an overview of the latest advances in genomic editing in these yeasts, their main
322 applications and the main challenges.
323 *Pichia pastoris:* reclassified as *Komagataella pastoris*, widely used today in the biotechnological field for
324 the production of heterologous proteins [36]. In Austria they demonstrated the integration of
325 cassettes with donor DNA without markers in the wild type strain of *P. pastoris*, which allows new
326 engineering strategies. They tested three variants of Cas9 (Sp Cas9, Pp Cas9, Hs Cas9) available, to
327 be evaluated in the pastorku70 strain of *P. pastoris*, they wanted to determine if they would need a
328 different level of expression. For the wild-type strain only Hs Cas9 was included and gave the Cas9/
329 gRNA expression plasmids a Geneticin resistance marker which proved to be a versatile tool for the
330 recycling of markers. The CRSIPR-Cas9 tool can be applied to modify the existing production strains
331 and also open the way for studies of complete genome modification without markers in *P. pastoris*.
332 In addition, it can be implemented for autonomous replication sequences (ARS) in classic knockout
333 cassettes to guarantee cassette maintenance, especially in cell cycles in the S / G2 phase where HR
334 predominates and, therefore, favor their integration[37].
335 In 2016 the same Austrian research group mentioned above, systematically tested more than 90
336 constructs containing different DNA sequences optimized with Cas9 codons, several gRNA
337 sequences and promoters of Pol III RNA and Pol II RNA (in combination with ribozymes) for the
338 expression of the gRNAs with different promoters of Pol II RNA for the expression of Cas9 and
339 gRNAs, in order to identify the system failures in *P. pastoris*. They managed to generate an optimized
340 system for this model which allows alter genes, introduce deletions of multiplexed genes and test the
341 targeted integration of homologous DNA cassettes [38].

342 *Saccharomyces pastorianus:* it is widely used for brewing and in recent years its interest has been related
343 to certain glycolysis processes. In the Netherlands, a method based on the CRISPR-Cas9 genome
344 editing was designed for the precise elimination of genes (SeATF1 and SeATF2) in *S. pastorianus*. The
345 cas9 gene expressed from a mobile genetic element was combined in combination with a plasmid-
346 transmitted gRNA expression cassette, the expression of a gRNA flanked with Hammerhead
347 ribozymes and delta Delta viruses using the TDH3 promoter dependent on RNA polymerase II
348 successfully led to the precise elimination of the four alleles of SeILV6 in the strain CBS1483, in
349 addition, the expression of two gRNAs flanked by ribozymes separated by a 10 bp linker in a
350 polycistronic matrix successfully led to the simultaneous elimination of SeATF1 and SeATF2 genes,
351 located in two separated chromosomes[39]. The compilation of these studies of editing of the genome

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352 by CRISPR-Cas in washes, allowed to consolidate a general vision of the advances of this technology
353 of showing versatility in editing and approaches, which has allowed the creation of a CRISPRi strain
354 collection for more than 99% of the genes needed for fermentative or respiratory growth [40].

355 The aforementioned studies on the genome edition in yeasts of industrial, biotechnological or clinical
356 interest are of great importance to understand the biology of fungal cells in fermentative processes in
357 the industry, important pathogenic processes for clinical studies or pharmacokinetic processes.
358 CRISP-Cas is a new methodology implemented in yeasts, there is still a lot to explore and learn.

359 **Development of various approaches for the use of the CRISPR / Cas system**

360 Since 2012, the CRISPR-Cas system has been used for gene editing and its use has been extended to
361 different species. In recent years, methodologies have been designed that use different CRISPR
362 systems depending on the modifications that are to be made in the genome, here we collect some of
363 the genome editing modifications (Table 1).

364 **Table 1 New CRISPR methodologies.**

Technology	Name	Functions	Reference
Di-CRISPR	Delta Integration CRISPR/Cas	Integration of biochemical pathways in one step, without markers for Cas9, by selecting repeated delta sites.	[41]
CasEMBLR	CasEMBLR	Multi-genomic integration loci facilitated by Cas9 from in-vivo assembled parts.	[42]
CRISPR-AID	CRISPRa, CRISPRi and CRISPRd	It allows to increase the activation of a gene to overexpress metabolic compounds. Several metabolic engineering targets can be used for optimization and the exploration of synergistic interactions between transcriptional activation, transcriptional interference and gene elimination can be used.	[43]
CRISPR-Cpf1	Cpf1	It allows to increase the activation of a gene to overexpress metabolic compounds. Several metabolic engineering targets can be used for optimization and the exploration of synergistic interactions between transcriptional activation, transcriptional interference and gene elimination can be used.	[44]
CRISPR-PCS	Division of chromosomes mediated for PCR	Improves the efficiency of chromosome division almost 200 times. Allowing to create new strains with specific traits for industrial applications and investigate the function of the genome.	[45]
dCas9-VPR	Cas9 with the chimeric activation domain VPR.	It allows performing transcriptional repression or transcriptional activation.	[46]
TAR-CRISPR	Recombination associated with transformation	It allows to isolate any chromosomal region by homologous recombination, between mammalian cells and yeasts such as *S. cerevisiae*	[47]
CRISPRm.	CRISPR multiplex system	Allows you to edit multiple loci simultaneously	[48]
CRISPRa	Activator	Allows transcriptional activation	[49]
mCRISTAR	CRISPR Cas9 y TAR	It allows the multiplexed substitution of promoters in groups of biosynthetic genes avoiding the regulatory elements of transcription, inducing gene expression	[50]
dCas9	Cas9 deactivate	Allows gene repression in a high efficiency repression site	[51] [52]

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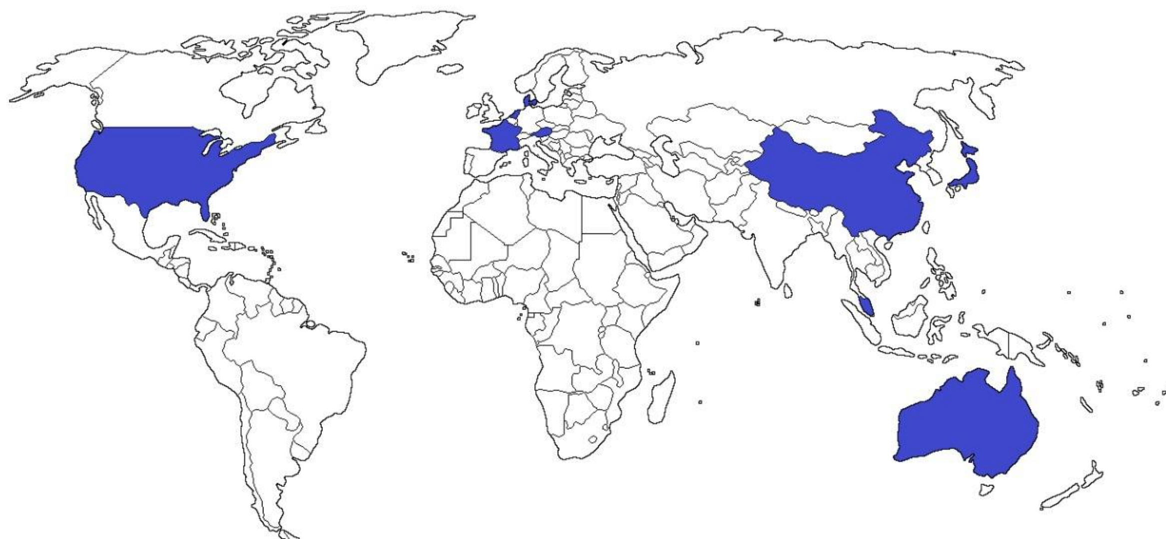
365 From the modifications made to CRISPR and its implementation in the edition of the genome in
366 yeasts, we found that the system is very dynamic, adapts to almost any experimental design and
367 can be implemented for any genetic modification that is desired; each of these variants improves
368 the efficiency of the system and facilitates the editing process.



369 Figure 1. Mundial distribution with information about CRISPR-Cas system in yeast.

370 ## 3. Conclusion

371 The CRISPR system is currently one of the most diverse genetic editing systems. It allows gene edition
372 in different organisms as long as an efficient computer design of the sgRNA sequences is available. It
373 will be implemented to achieve the objective of the study, be it to eliminate a gene, make point
374 mutations, express a gene or do the aforementioned in several genes simultaneously. On the other
375 hand, the new kits generated for this system have certain limitations. For example, the Easy-Clone-
376 MarkerFree kit designed in Denmark, where they obtained several integration cassettes
377 simultaneously into the yeast genome.

378 One of the major concerns in the use of CRISPR-Cas is the management of modified strains, in most
379 cases, the authors use control points in which they can elucidate the changes made, and in some cases,
380 indicate when they were lost. However, in some investigations the management of these strains is
381 not clear, although CRISPR / CAS is a technology that has provided many benefits in the genome
382 edition thanks to its highly specificity and efficiency compared to the edition of TALENS and SINES.
383 Its rapid evolution leaves little time for ethical and biosecurity controls, since organisms published
384 by this technology could alter ecosystems if they are not properly managed.

385 As underlined in these articles, CRISPR is a widely used tool for better industrial processes, either to
386 increase production or to eliminate some toxic compound that is generated during the production
387 process of some molecule or industrial product in the yeasts are needed to synthesize or ferment
388 them as it is in the case of alcoholic beverages, in addition, in the clinical area this tool has facilitated
389 the study of several genes involved in signaling pathways that confer the yeast, either resistance
390 treatment pharmacology, or confers pathogenicity on the host. Allowing thus generate alternatives
391 for the industry and in clinic to problems that affect the human being.

392 **Supplementary Materials:**

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393 Methodology

394 Search strategy: the PubMed, Lilacs and google academic databases were consulted. The inclusion
395 criteria were all articles that included the CRISPR /Cas system in yeast, date restriction was not used,
396 articles were consulted only in English and Spanish, searches were made through MeSH terms and
397 Boolean operators, the terms of exclusion were other microorganisms different from yeast and other
398 genome editing methodologies.

399 Selection of the articles: it was carried out according to the PRISMA methodology, it consists of a
400 minimum set of elements, based on evidence, to help to present reports of systematic reviews, and it
401 is useful for the critical evaluation of articles. The articles were evaluated and verified independently
402 by four evaluators taking into account the title, summary, and reading of articles (See graphic prism
403 Annex 1).

404 Analysis of articles: The articles were classified according to the type of study, yeast species and
405 CRISPR / CAS technology type. The data was tabulated in the Excel program.

406

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412

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