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CRISPR-Cas9 and its application potential in the brewing industry

Over the last years, the CRISPR-Cas9 system has developed into the de facto standard gene modification technique in exploratory research. However, owing to its ease of usage and efficiency, CRISPR-Cas9 has also increasingly been exploited in the food industry while several applications have been reported for the brewing sector. Based on these developments, the present review aims at providing an overview of both the molecular background and function of the CRISPR-Cas9 system. This is followed by application examples of interest to the brewer. In the last part, the EU regulations and current scholarly discussion on genetically modified organisms are briefly summarized.

Descriptors: CRISPR-Cas9, gene manipulation, application, brewing, yeast, EU legislation

1 Introduction

Yeast strains, like *Saccharomyces cerevisiae*, used in commercial brewing or baking applications, have been traditionally improved by random mutagenesis or classical breeding. In 1995, *John Hammond* pointed out the limitations of the existing brewing yeasts and presented numerus genetical modification tools for improving *Saccharomyces* strains [1]. Within the next five years, the number of available genetic engineering methods for *S. cerevisiae* increased rapidly (reviewed in [2]). The brewing industry also applies genetical modification techniques in order to enhance or reduce certain yeast properties or behavior. For example, to create a yeast strain suitable for a lower alcohol production, Nevoigt and co-workers designed a strain to shift carbon flux from alcohol production towards glycerol [3]. Application of genetical engineering tools also enables yeast to produce yeast-foreign active flavor compounds, like raspberry ketones, relevant for the alcoholic beverage industry [4].

There are not many techniques which revolutionized their field overnight. CRISPR-Cas9 is such a rare example and the current de facto standard for gene editing. The development of this method consequently resulted in the award of a Nobel prize for Emmanuelle Charpentier and Jennifer Doudna in 2020 [5]. The major advantage of CRISPR-Cas9 is the flexibility of the system, allowing gene targeting by simple generation of a custom guide RNA. This enables the easy introduction of a range of genetic modifications. Simple gene knock-outs can be achieved, or with additional usage of DNA templates, precision mutations ranging from point mutations to generation of fusion proteins (reviewed in [6]). While CRISPR-Cas9 exhibits a high off-target DNA binding, it only has a low off-target DNA cleavage rate [7–9]. The flexibility and high specificity renders this technology not only interesting in fundamental research but also for medicinal (reviewed in [10]) and general food applications (reviewed in [11, 12]).

Gene editing techniques for the production of food and feed has since been subject to legal regulations. The European Union describes in the 90/220/EEC council directive a genetically modified organism (GMO) as an organism with altered genetic material in a way which cannot naturally occur [13]. Once classified as GMO, products need to seek authorization by the European Food Safety Authority (EFSA) [14]. The allowed methods to create genetically altered organisms not classifying as GMO were narrowed by Judgment of the European Court of Justice in its ruling in case C528/16 [15]. A Commission study, evaluating the justification of this ruling, was published last year in April [16].

In summary, the following review wants to give a brief introduction into the molecular mechanisms behind CRISPR-Cas9 and recent applications in the brewing industry. In the last chapter a summary of the legal framework of regulations pertaining to food related GMO in the European Union together with the current state of scholarly discussion is given.

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2 Molecular Background and Function of the CRISPR-Cas9 System

For decades, clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated (Cas) systems were terra incognita. While studying the *Escherichia coli* "alkaline phosphatase isoenzyme conversion protein" (iap), Ishino and his co-workers discovered a neighboring DNA sequence forming an array of short 20 to 40 bp repetitive sequences ("repeats") that were interspaced by nonrepetitive "spacer" sequences [17]. Almost

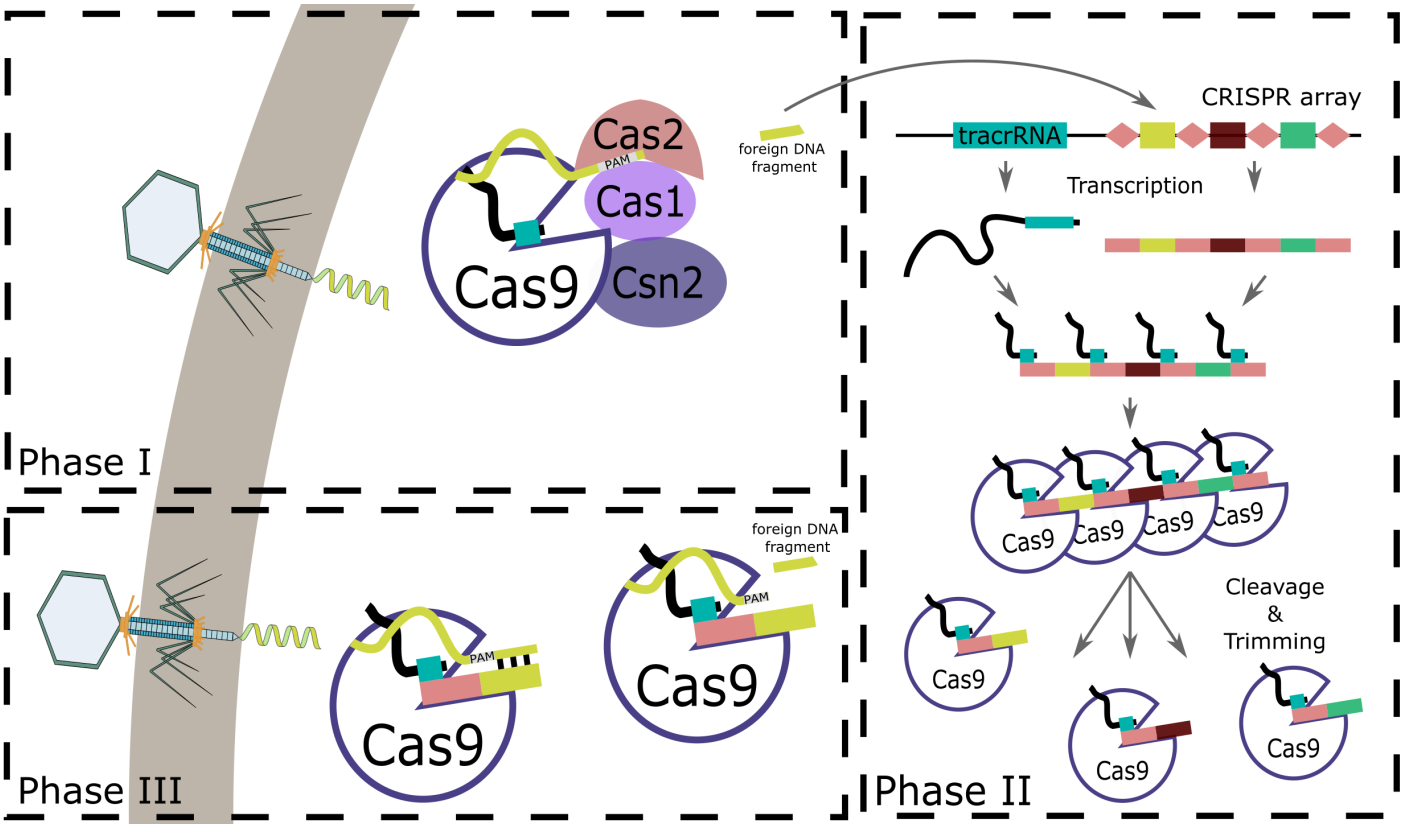

Fig. 1 **The three phases of the type II CRISPR-Cas9 immune system. For reasons of visual clarity, the stoichiometry of the constituents of the protein complexes is not taken into account. In phase I the bacterial cell is infected with foreign double-stranded DNA. This DNA is preprocessed (not shown) and bound by Cas1, Cas2, Csn2, and Cas9. The PAM domain located on the protospacer licenses Cas9 to cut the foreign DNA. A fragment of this foreign DNA is then integrated by the Cas1-Cas2 integrase complex into the CRISPR array as spacer. Simultaneously to integration, the flaking repeats are synthesized. In phase II the CRISPR array is transcribed as pre-crRNA. The tracrRNA is transcribed as well. The tracrRNA binds to the pre-crRNA repeats and moderates the binding of Cas9. Finally, RNAse III cleaves the pre-crRNA into crRNAs. The crRNAs get trimmed so that a spacer sequence (guide) of 20 nucleotides remains. This creates a functional Cas9 system. In phase III a new infection with previous encountered DNA occurs. If a similarity between foreign DNA and guide is detected, together with presence of a PAM, a DNA double strand break is initiated by the Cas9 system**

two decades later, with more genomes being sequenced and (publicly) available, computational scientists uncovered that the repetitive sequences were also present in numerous Eubacteria and Archaea. Even more striking, the spacer sequences were highly identical to exogenous mobile genetic elements such as plasmids, bacteriophages and transposons [18–20]. Thus, it was hypothesized that these sequences might be an adaptive system that protects the bacterial cell from foreign genetic elements [21]. In 2007, it was confirmed that the CRISPR-sequences and the associated Cas proteins interfered directly with a bacteriophage infection. In addition, new spacer sequences were integrated into the CRISPR array with every new bacteriophage encounter. This behavior was consistent with a role as adaptive immune system against invaders [21–23]. Since then, Scientists have been inspired to apply CRISPR-Cas systems for biotechnological purposes.

To understand the mechanism behind CRISPR-Cas gene editing, it is important to understand how this bacterial adaptive immune system functions. Two CRISPR-Cas classes comprising a total of six distinct types are currently known [24, 25]. These classes and types differ in the recognition and cleavage mechanism of foreign DNA. Generally, the adaptive immunity occurs in three subsequent stages: I) spacer acquisition, II) transcription of CRISPR RNA, and III) interference with invaders [26–28]. In the present article, the focus will be mainly on the type II CRISPR-Cas9 system found in *Streptococcus pyogenes*, as most of the gene-editing applications are based on this system (see Fig. 1).

The spacer acquisition occurs when foreign DNA is entering the cell. In the first step, the DNA repair machinery of the host cell is co-opted into the CRISPR mechanism as it detects free ends of double stranded DNA (dsDNA) [29]. In this instance, it is interesting to note that bacterial chromosomes are circular (with exceptions, reviewed in PMID: 10802162) and free dsDNA ends only rarely occur e.g. in case of DNA double strand breaks. The incorporation of such sites with free dsDNA from the host genome into the CRISPR array, would lead to auto-immunity and cell death (reviewed in [30, 31]). Even if free double strand DNA in the host occurs, CRISPR array incorporations are further believed to be constrained by the occurrence of so-called Chi elements in the host DNA. This is suggested to help the CRISPR system to differentiate self from non-self DNA [29, 32]. In gram-positive bacteria (like *S. pyogenes*) free ends of dsDNA are detected and processed by the helicase/nuclease AddAB (called RecBCD in gram-negative bacteria). The exact mechanism by which these AddAB degradation products are utilized in protospacer acquisition is currently unknown. There is also evidence for the existence of alternative pathways independent of AddAB (reviewed in [33]). In the next step, these so-called

protospacers are bound by the Cas1-Cas2 proteins in complex with Csn1 and Cas9 [34]. Target cleavage by Cas9 subsequently takes place, however only if a protospacer adjacent motif (PAM) is present on the protospacer. PAMs are short stretches of 2–5 nucleotides, located directly next to or up to 3 bp after the spacer sequence [20, 35–39]. The associated PAM-sequence for *S. pyogenes* Cas9 is 5`-NGG-3` [35, 38]. The occurrence of PAMs on the protospacer is important as these PAMs are not incorporated into the host genome to prevent cleavage of the CRISPR array [35, 36]. After cleavage, the processed spacer is incorporated into the CRISPR array by the Cas1 and Cas2 protein complex [40–44]. The Cas1-Cas2 complex contains two DNA binding sites, one that binds the protospacer, the other binds to the CRISPR array. During insertion, the spacer is being flanked on both sides by a conserved repeat sequence ([45], also reviewed in [33]). It is noteworthy to mention that in type II systems, the integration occurs at the upstream side of the array, prioritizing the protection of the bacterium against the most recent invaders [22].

The second step is the generation of CRISPR RNAs (crRNAs) by transcription of the CRISPR array. The array consists of all previously acquired spacers and the flanking DNA sequence repeats in sequential order. The complete array is transcribed, generating a single precursor crRNA (pre-crRNA). At the same time, the so-called trans-activating crRNA (tracrRNA) is transcribed. The tracrRNA binds to the sequence repeats (flanking the spacers) of the pre-crRNA and is required for crRNA maturation. Cas9 binds to the pre-crRNA:tracrRNA complex and promotes its processing [46]. Then the pre-crRNA gets cleaved by RNase III into several crRNA fragments. These fragments get subsequently trimmed by as of yet unknown nucleases so that a 20 nucleotide targeting spacer (guide) RNA next to the repeat sequence remains [46]. This guide RNA is based on the (previously encountered) invader-sequence and thus facilitates identification of foreign DNA with significant complementarity to the guide.

During the final third and final step, when the invader re-infects the bacterial cell, the foreign nucleic acid sequence including a PAM is detected and cleaved by the Cas9:tracrRNA:crRNA complex, effectively halting the infection. Cas9 contains two endonuclease domains: the HNH domain and the RuvC-like cleaving domain [21]. When binding the tracrRNA, Cas9 undergoes a conformational change which creates a channel through the protein [47, 48]. When DNA passes through this channel, Cas9 scans this DNA for a high similarity with the guide RNA. The PAM sequence assists in this recognition and licenses the cleavage of the DNA [47–50]. It is noteworthy to mention that in the absence of the PAM sequence, even with full complementarity between the guide RNA and the target, the Cas9 enzyme will not cleave the sequence [51].

The insight that CRISPR-Cas systems can cleave DNA in a target-specific manner and the fact that the CRISPR-Cas system II only needs a single cleaving enzyme (Cas9) instead of a whole complex, proved to be extremely powerful [52]. In contrast to previously applied genome editing technologies, CRISPR-Cas9 can be easily adapted to target specific DNA sequences by adjusting the guide RNA. The introduction of double strand breaks (DSB) at specific sites adjacent to a PAM by Cas9 is exploited in genome editing

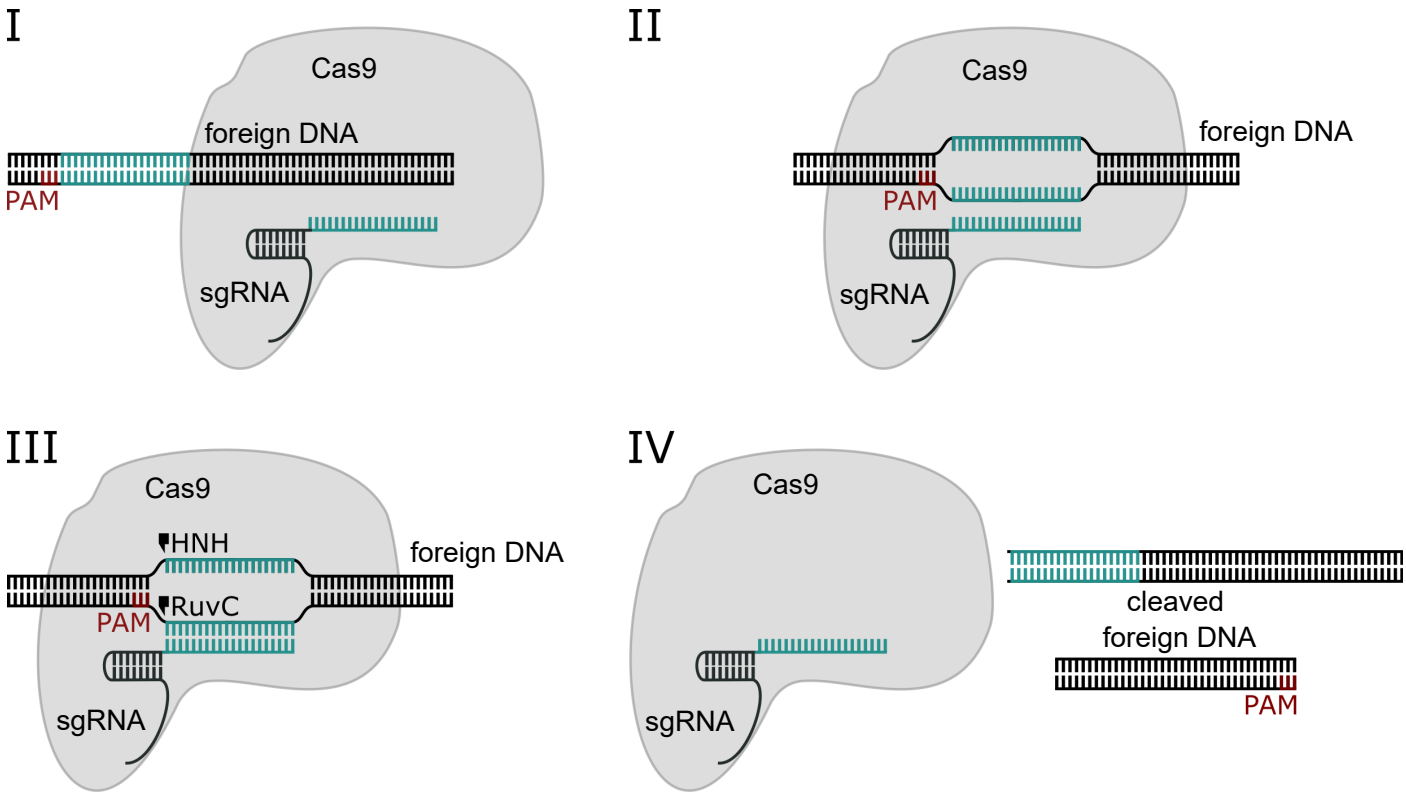

Fig. 2 **The endonuclease mechanism of CRISPR-Cas9. (I) The (foreign) DNA passes through the Cas9:sgRNA (tracrRNA:crRNA joined by a loop) complex. (II) A complementarity between the DNA and the 20 nucleotide guide sequence as well as the occurrence of a PAM structure is detected. (III) The two endonuclease domains HNH and RuvC-like introduce a single strand break each. (IV) The foreign DNA is cleaved between the PAM and the gene sequence complementary to the guide sequence**

[38]. In general, DSBs are restored by non-homologous end joining (NHEJ), an error-prone cellular DNA repair mechanism which often leads to random mutations. NHEJ can cause nucleotide insertions or deletions, which potentially result in frameshift mutations. These frame-shift mutations can introduce premature stop-codons downstream of the cleavage site, leading to null-mutations [53, 54]. Although DSB are repaired most frequently through NHEJ in somatic cells [55], the unpredictable outcome is not always suitable for gene-editing purposes. This is remedied by an alternative DNA repair system called homology-directed repair (HDR). In contrast to NHEJ, HDR requires a homologous DNA sequence at the cleavage site (e.g. from a sister chromatid during DNA replication). This homologous sequence acts as the instructive template used in the repair of the DSB. HDR is therefore virtually error-free and the preferred mechanism to repair DSB during the S and G2 phases of the eukaryotic cell cycle (reviewed in [56]). By introducing a template containing a custom sequence, which is flanked by the homologous regions up- and downstream of the cleaving site, the integration of precise changes into the genome can be achieved [53, 57]. Examples for such kinds of changes are point mutations to alter single amino acids in the resulting protein or the generation of fusion proteins containing reporter tags.

In current research, the CRISPR-Cas9 system type II is regularly applied. As described above, the mechanism consists of two components: a DNA cleaving enzyme (Cas9) and the tracrRNA:crRNA heteroduplex. One major simplification of the system was the replacement of the tracrRNA:crRNA heteroduplex by a single-stranded structure called single guide RNA (sgRNA). The sgRNA is a single molecule that resembles the tracrRNA:crRNA duplex with the addition of a loop that connects the tracrRNA and the crRNA permanently [38, 58] (see Fig. 2). The sgRNA retains two essential features: a 5` 20-nucleotide guide domain for DNA targeting and a 3`domain for interaction with Cas9.

When applying CRISPR-Cas9 for genetic engineering, the essential components to modify the genome need to be delivered into the cells. HDR is the preferred DNA repair pathway in yeast [59]. Therefore, yeast cells are often co-transformed using a plasmid and a linear DNA molecule. From the plasmid, Cas9 and a cloned sgRNA are co-expressed, leading to the directed cleavage of the target DNA. The linear DNA molecule is used as a template to repair the DNA strand via HDR and enables introduction of precise and defined mutations. The CRISPR-Cas9 system has successfully been used in yeast to generate gene deletions [60], gene disruption [57] and gene integration [61]. The efficiency to introduce template driven mutations is limited by their size, with lower efficiencies encountered when using longer DNA sequences [62]. Furthermore, the specificity of the guide allows for multiplexed systems, in which the plasmid encodes for multiple sgRNAs, allowing the simultaneous editing of several genes [63].

When performing gene editing, off-target action (i.e. cleavage of unintended DNA sequences) is always of major concern. CRISPR-Cas9 is no exception to this and off-targeting has been previously described [7–9]. However, while off-target DNA binding using CRISPR-Cas9 is quite promiscuous, off-target cleavage is rare [7, 8, 38, 64]. The risk of off-targeting can be minimized utilizing paired sgRNAs together with a mutated form of Cas9 [65, 66]. In these forms one of the endonuclease domains of Cas9, RuvC-like (D10A) or HNH (H840A), is mutated. This results in a Cas9 nickase that generates single strand breaks [38, 53, 57, 67]. The introduction of two single strand breaks improves the specificity of genome editing as it requires the binding of two sgRNAs. If these single strand breaks are in close proximity to each other they can easily evolve into a DSB [65, 66, 68, 69]. A more recent study found that the HNH cleavage site is more efficient than the RuvC-like site, thus the application of Cas9n D10A nickases are currently preferred [70]. However, competent design of the sgRNAs is key to mitigate the nontarget interactions of Cas9. Potential off-targeting of the sgRNAs can be assessed through databases like E-Crisp and Off-spotter [71, 72].

3 CRISPR-Cas9 Applications in Brewing Science

While gene editing approaches in brewing related applications have been reported, they mostly use traditional techniques. Increased applications of CRISPR-Cas9 are evident in the literature of recent years. However, publications specifically mentioning brewing specific applications for CRISPR-Cas9 in yeast, cereals and hops are still rare.

CRISPR-Cas9 research employing cereals are mostly related indirectly to the field of brewing and focus on the mitigation of climate change effects, pathogen resistance, abiotic stress tolerance, improved yields and nutritional quality (reviewed in [73–77]). On a topic directly relevant to the brewing industry, a single study was recently published by *Hisano* et al. [78]. There, the influence of the proteins Qsd1 and Qsd2 on barley dormancy, an important phenomenon in malting, was investigated. The loss of function in the Qsd1 and Qsd2 genes resulted in prolonged barley grain dormancy. Qsd2 appeared to be dominating Qsd1 in terms of dormancy responses. Also, Qsd2 was suppressing germination under cold conditions, however Qsd1 seemed to mitigate this [78]. A further development on the plant side of the brewing sciences is the development of a CRISPR-Cas9 procedure for hop [79].

Protocols for editing *Saccharomyces cerevisiae* by CRISPR-Cas9 have been established and applied in a myriad of publications, although mostly for fundamental research purposes outside the field of brewing (reviewed in [57]). Although, a protocol for *Saccharomyces pastorianus* was described recently [80].

The beer market is saturated and there is a demand for new flavors created by new yeasts [81, 82]. To create new varieties of brewing strains and thus new beer flavors, yeast hybrids were created in several laboratory experiments [81, 83]. An increased phenolic off-flavor production, such as 4-vinyl guaiacol (4-VG), was found in most of the newly created yeast hybrids [81, 83, 84]. The removal of these unwanted flavors by crossing-back with one parental strain, which does not produce the off-flavors, was described [85, 86]. However, *Mertens* et al. found this method to be unfavorable in terms of time investment and cost [82]. Therefore, instead of back-crossing, a CRISPR-Cas9 approach was employed to create a loss-of-function mutation in the FDC1 gene [82]. This gene has shown a correlation with the ferulic acid decarboxylation ability in yeast [87]. By introducing a point mutation in FDC1 Mertens et al.

created yeast mutants which lost their capability to convert ferulic acid to the phenolic off flavor 4-VG. In a validation experiment, 17 different flavor-active metabolites of the mutant and the wild type were compared. Except for the targeted mutation, no significant differences in flavor-active metabolites between the wild type and the mutant were detected [82].

Another approach to create a new variety of yeast suitable for beer production was conducted by *Krogerus* et al. [88]. Eight yeast strains were selected based on their β-lyase activity. This enzyme is responsible for the release of volatile sulfur compounds from their non-volatile precursors [89], which are associated with tropical flavors. Yeast strains with high β-lyase activity which were additionally lagging in off-flavor production were chosen to create mating-competent yeast cells. By introducing a double strand break in the mating-type protein gene locus with subsequent knock-out, a sterile industrial transformant with a specified mating type was created. The created mating type cells were stable and capable to mate with cells of opposite mating type to form new hybrids. The newly created hybrids showed heterosis (i.e. advantages in the hybrids as compared to the parent strains) in key attributes like fermentation performance and aroma formation [88].

To tackle the increasing prices and flavor inconsistencies of hop plants, researchers aimed to develop a *S. cerevisiae* strain capable of expressing monoterpenes, adding a hoppy flavor to the finished beer [90]. Prior to this study, the key monoterpenes responsible for a hoppy flavor were identified as linalool and geraniol [91]. Linalool and geraniol are catalyzed from geranyl pyrophosphate (GPP) by the monoterpene synthase [92]. *Denby* et al. hypothesized that by implementing monoterpene synthases into *S. cerevisiae*, the yeast will utilize the generally limited but naturally build GPP into linalool and geraniol [90]. Therefore, monoterpene biosynthesis pathway genes were designed by a combination of different genetic elements. With CRISPR-Cas9 strains were constructed according to the designed monoterpene biosynthesis pathway. Created strains were tested in a fermentation which was analyzed via LC/MS, HPLC and GC/MS. A taste panel evaluated the beer produced by the optimized strains and determined a range of hop flavors. Denby et al. concluded, that yeast-built monoterpenes give rise to hop flavor in the finished beer and provide more consistency than traditional hopping [90].

A further application of CRISPR-Cas9 was described in the production of lactic acid by *S. cerevisiae*, suitable for sour beer fermentation. Alternative production methods for sour beer are currently gaining more momentum to satisfy increasing consumer demand [93–95]. Traditional fermentation uses a spontaneous process where wort is inoculated by environmental exposure, alternative techniques utilize deliberate microbial inoculation, e.g. kettle sour [96, 97]. In general, *S. cerevisiae* is suitable for the production of lactic acid due to its simple nutrition requirements and its relative high acid tolerance. Taking advantage of these benefits, *Mitsui* et al. constructed a lactic acid producing yeast strain combining a global metabolic engineering strategy and a CRISPR integration system [98]. First, the yeast tolerance to low pH was improved by genome evolution under low pH stress conditions. In a similar matter a lactic acid tolerant mutant was created. In the last step the obtained acid tolerant mutant was metabolically engineered to produce D-lactic acid. The authors hypothesized that the increased tolerance of lactic acid is a results of the increased ATP synthesis, the H^+-ATPase controlled cytoplasmic pH level, the active protection of unaggregated proteins and refolding of denatured proteins, the enhanced degradation of reactive oxygen species and a change in the cell wall composition [98].

However, CRISPR-Cas9 was not only used in the generation of new yeast strains, but also in fundamental research applications in which cellular systems relevant for beer fermentation were studied, i.e. maltose metabolism and aroma compound formation.

The maltose metabolism of *S. pastorianus* was studied by *Brickwedde* et al., applying CRISPR-Cas9 [99]. *S. pastorianus* originated from a hybridization event between *S. cerevisiae* and *Saccharomyces eubayanus* [100] with maltose uptake in *S. eubayanus* being not well studied. Brickwedde et al. investigated the contribution of individual, putative maltose-transporter genes (SeMALT) in *S. eubayanus* [99]. Four suspected genes for the expression of putative maltose transporters (SeMALT1, SeMALT2, SeMALT3 and SeMALT4) were identified in subtelomeric regions. To explore their impact on maltose up-take, systemic overexpression of these genes in a maltose transporter negative strain were conducted. This showed the predominant responsibility of SeMALT2 and SeMALT4 for maltose uptake in the used *S. eubayanus* strain. Although SeMALT1 and SeMALT3 demonstrated an impact on growth on maltose in *S. cerevisiae*, the genes were not able to compensate for the loss of SeMALT2 and SeMALT4 in *S. eubayanus*. The researchers conclude that they neither suspect nor exclude *S. eubayanus* as potential donor for the brewing relevant capability of maltose up-take in *S. pastorianus*, because it is unlikely that the used *S. eubayanus* strain contributed the *S. eubayanus* subgenome of *S. pastorianus* [99].

Krogerus et al. investigated the maltotriose and starch utilization in various *S. cerevisiae* and a single *S. pastorianus* strains [101]. Out of 15 strains carrying the gene coding for glucoamylase S1 (STA1), only five were capable of utilizing maltotriose and starch. Genome sequencing revealed a 1162 bp deletion upstream of STA1 in the strains unable to utilize maltose and starch. To confirm whether this deletion inhibits the transcription of STA1, a CRISPR-Cas9 induced deletion of the 1162 bp region was performed. The results confirmed a linkage between decreased diastatic ability and the 1162 bp deletion upstream of STA1. Krogerus et al. concluded that STA1 plays a key role in enabling the maltotriose utilization and could be exploited for strain development where maltotriose utilization is desired [101].

An investigation by *Dank* et al. tested if the aroma profile of beer is influenced by esterase activity. It was examined whether a high esterase activity is the limiting factor for the abundance of esters and therefore the key for the aroma profile of fermented alcoholic beverages in *S. cerevisiae* [102]. Mutants lacking the capability to synthesize the esterases isoamyl acetate hydrolysing esterase 1 and the temperature shock-inducible protein 1 were created using CRISPR/Cas9. The esterase activity and aroma profiles of the created mutants differed significantly from the wild type. These results suggest a link between esterase activity and aroma production. The authors hypothesize that the identification of the regulatory

system for aroma production could lead to the ability to precisely engineer the aroma production during fermentation [102].

4 CRISPR-Cas9 and European Food Law

To establish appropriate GMO handling, several EU regulations are in place e.g., 2001/18/EC regulating the environmental protection from unwanted GMO release [103] and (EC) No 1830/2003 determining the correct labelling of GMOs in food or feed products [104].

The regulation EC No 178/2002 provides the underlying principles and requirements of the European food law. This regulation also established the European Food Safety Authority (EFSA). The Authority consists of a management board, an executive director, an advisory forum and a scientific committee and panels. The EFSA is supposed to provide scientific opinions for legalization of feed and foodstuff as well as other matters within its mission (e.g. animal health, welfare and plant health) to the member states. Therefore, the authority promotes and coordinates the development of risk assessments, collects, analyses and summarizes scientific and technical data within its operation field. The EFSA also establishes a system of information transfer to the European member states. Furthermore, the EFSA is responsible for the authorization of genetically modified (GM) food and feed [14]. More specifically, the GM food and feed regulation (EC) No 1829/2003 describes the "one door one key" authorization procedure by the EFSA which applies to any GMOs for food or feed usage and food or feed containing GMOs or ingredients produced by GMOs. The same authorization criteria are in place for cultivation of GMOs. In this context, the 'one door one key' authorization means that for the authorization of a GMO for cultivation, food or feed only a single, common application has to be filed since food, feed and GMO for cultivation have to adhere to the same standards [105]. EFSA authorizations are granted for 10 years. A new authorization can be requested between 3rd and 9th year of market availability [106]. It also declares that food or feed containing less than 0.9 % of EU authorized GMO material and 0.5 % of not authorized GMO material can be classified as non-GM food or feed. The regulation discriminates between food or feed "from" GMOs and food or feed "with" GMOs. Food or feed from GMOs need to seek approval from the EFSA. On the other hand, food or feed produced with the help of genetically modified processing aids, can enter the European market without an additional authorization step. For example, a product obtained from an animal, which was fed with GM-feed or got treated with genetically modified medical products, is not considered an GMO itself and is not required to seek authorization nor requires a specific GM label [106]. In other words: Beer produced by GM yeast, hop or malt, can be considered as a non-GM food provided the GMO DNA is removed below the threshold of 0.9 % or 0.5 % according to their authorization status (authorized or unauthorized, respectively).

The European Community council directive 90/220/EEC from the 23rd of April 1990 defines a genetically modified organism as an organism with altered genetic material in a way that cannot naturally occur [13]. There are 3 types of naturally occurring mutations: genome mutations, point mutations and chromosome mutations. A genome mutation results in an altered number of chromosomes compared to the wild type. A point mutation describes the addition, deletion or change of a single base pair in the DNA. A deletion or addition of a single base pair results in a frame shift during transcription by RNA-polymerase and the protein cannot be translated in a functioning way. On the other hand, the exchange of a single base pair can result in a silent mutation, where the protein is built and functions as intended, or can result in a non-sense or mis-sense mutation. A non-sense mutation introduces a premature stop codon, a mis-sense mutation causes the amino acid sequence to change and the translated protein to be potentially dysfunctional. Chromosome mutations include deletion, duplication, inversion and translocation of chromosomal areas [107].

In the council directive 90/220/EEC different gene modifying techniques creating a GMO are listed e.g., DNA-recombination applying a vector system, artificially occurring cell fusion and direct introduction of DNA which was created outside of the organism. The performance of in vitro fertilization, conjugation, transformation, polyploid induction or any other natural process do not create a genetically modified organism [13]. Due to the ruling in the case C-528/16, by the Judgment of the European Court of Justice (grand Chamber) from the 25th July 2018, naturally occurring mutagenesis is categorized as a technique to create recombinant nucleic acids or GMOs, which are excluded from the GM food and feed regulation (EC) No 1829/2003 [15]. Mutagenesis is generally defined as a mutation introducing process [108]. The ruling in case C-528/16 declares that mutagenesis may not transfer foreign DNA into a living organism. Furthermore, mutagenesis must occur randomly and is not allowed to be directed by site-directed nucleases or oligonucleotides. By this understanding, a microorganism with point mutations created by a CRISPR-Cas9 system needs to be classified as GMO [15].

There are several articles criticizing these EU regulations on GM food and feed. For example, Tadesse Firkre Teferra criticizes whether the safety regulations are justified in regards to the world hunger problem and the potential solutions GM food and feed could provide in this field [109]. *Eriksson* et al. wrote a series of three articles arguing to overcome the stalling innovation without neglecting the precautions and proposed options to reform the legislation on GMOs [110–112]. Further, *Christiansen* et al. debated the justification of GMO restriction by balancing the argument for and against a GMO authorization within the European market [113]. Arguments in favor of a GMO authorization included for example the decreased usage of pesticides, a possible solution to the world hunger crisis and liberty of the people to choose individually. Arguments for GMO restriction range from the harm to non-target species and negative health effects, to crop failure resulting in the collapse of the food system and possible human extinction. Christiansens and co-workers concluded that the current regulations are in general not justified and that due to the very low numbers of authorizations given, the pre-release authorization process needs to be reformed [113].

By council decision (EU) 2019/1904 of the 8th November 2019 the Commission was requested to conduct a study determining whether the judgment in case C-528/16 is appropriate in regards to novel gene engineering techniques [114]. On the 29th of April 2021 the

commission released a staff working document about the study on the status of new genomic techniques under Union law and in light of the Court of Justice ruling in Case C-528/16 [16]. The study recognizes similarities between conventional breeding and cisgenesis. Cisgenesis is a combination of breeding techniques and biotechnology in order to transfer genes between two related species and to speed up the breeding process [115]. Off-target mutations introduced by site directed nucleases or cisgenesis are regarded as being of the same type as created by conventional breeding. The commission finds the detection of unauthorized genome-editing unlikely since the mutation could also have been created by conventional breeding techniques. There are no screening techniques available to distinguish such a GM-product. The EFSA found an injustice since similar products exhibiting similar risk profiles have different levels of regulations. One being obtained by conventional breeding techniques, while the other was created by genome editing techniques and cisgenesis. A follow-up study is to determine whether the law needs to be adapted. Further, the study estimates about 30 GM-plants, animals and microorganisms altered with CRISPR could reach the European market within the next 5 years. By 2030 this number could increase to over 100 products [16].

5 Conclusion

CRISPR-Cas9 allows straight-forward genome editing as it only requires a simple modification of the guide RNA. Thus it is much easier to design the required molecules as in comparison to other gene-editing techniques like Zinc finger nucleases (ZFN) and Transcription activator-like effector nucleases (TALENs) which require considerable protein engineering efforts [116]. CRISPR-Cas9 also allows the introduction of changes which can mimic naturally occurring mutations without leaving remnants of foreign DNA behind. Thus, CRISPR-Cas9 enables the production of "gene-edited" or "non-transgenic" food as opposed to "genetically modified" food by classical gene editing technologies (which can contain transgene products). Under the current Judgment of the Court (Grand Chamber) in case C-528/16 organisms modified by CRISPR-Cas9 must be considered as GMOs. However, the current discussion around the EU regulation justification [117], flamed by the recent report about CRISPR-Cas9 by the EFSA could lead to changes in the existing regulations.

As mentioned above, since the arrival of CRISPR-Cas9, genetically edited organisms are simple to generate. Although their legal standing might radically change in the future, their application in food is also dependent on consumer acceptance. Data on this topic is limited thus far, but it is suggested that consumers are more willing to consume CRISPR-Cas9 edited (non-transgenic) food than genetically modified food products [118–120]. A recent paper by Ferrari and co-workers show that young consumers are, in general, more in favor of gene-edited food and those consumers with a medical or more hard-scientific background tend to know more about gene-editing methods and were less concerned about gene-edited food overall [121]. Although both genetic modification and gene-editing technologies are treated equally by European legislation, the majority of young consumers are less supportive of similar labeling policies for genetically modified and gene-edited food, suggesting that consumers differentiate between genetically modified and gene-edited food products [121, 122].

In the light of the recent legal developments and a seemingly increased consumer acceptance of gene-edited food, we thus expect an increased application potential of gene editing methods like CRISPR-Cas9 in the food, beverage and feed industry. Further, also an increase in application in fundamental brewing yeast research to elucidate the biology behind metabolomic traits (e.g. aroma compounds formation, carbon metabolization) and beneficial phenotypes (e.g. flocculation) is to be expected.

6 References

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