Review Paper:

CRISPR edited Microbes and their Industrial Potential Review

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Abstract

The CRISPR is Clustered Regularly Interspaced Short Palindromic Repeats, these repeated DNA sequences are found in the prokaryotes genome which is used as a tool to detect viral infections. CRISPR associated protein 9 i.e. Cas9 and the CRISPR together are forming the novel platform in the field of biotechnology. Nowadays, industries such as biotech, pharmaceuticals, agriculture, chemical, etc. are using highly engineered industrial strain.

They are used in targeting chromosomes, genetic manipulation, sequencing, gene replacement, embryonic stem cell manipulation and many more. The organisms modified by this CRISPR/Cas9 involved a huge diversity of targeting vectors including bacteria, yeast, fungi, algae (cyanobacteria, microalgae). CRISPR/Cas9 is a new generation genome engineering technology widely used in molecular genetics due to its ease, simplicity, efficiency and versatility.

Keywords: CRISPR, Cas9, Biotechnology, Microorganisms.

Introduction

A CRISPR immunity phenotype is genetically encoded by a so-called CRISPR locus (Clustered Regularly Interspaced Short Palindromic Repeats)—an array of repetitive and unique sequences (repeats and spacers respectively), both of which are typically around 30 nt in length[51]. These repeats and spacers are introduced in the bacterial cell by the phage infection which is used as a memory to combat future infections. As new spacers are added at the so-called leader-end of the locus, which is the sequence that contains the CRISPR promoter; CRISPR loci form an inverse chronological record of previous infections from the leader to the trailer end of the locus[50].

Apart from the genetic CRISPR memory, a functional CRISPR-Cas immune system also requires a set of CRISPR associated genes (cas genes), which encode the protein machinery required for carrying out the immune response[19]. Some researchers thoroughly discussed CRISPR/Cas technology and its biology[52,53]. Industrial microbiology plays a key role in the transition towards a more sustainable industry to produce food and feed ingredients, bio-based materials, biofuels and direct synthesis of cosmetic and pharmaceutical compounds[23].

Based on the notable publications on the internet, it has been researched that about 200- 300 papers have been published in first 6 months of 2019 that depict intense studies which are being performed on microbes of industrial significance using CRISPR/Cas technology. There has been an increasing interest in improving microbial cell factories through metabolic engineering approaches using CRISPR/Cas9 technology[18]. The efficiency and versatility offered by CRISPR tools have shown great potential in rewiring the metabolic network of host cells to enhance their production of metabolites used in various areas of industrial biotech ranging from applications as biofuels to chemical building blocks and pharmaceutical[13].

Earlier methods for genetic modification were not cost-effective, time-consuming and tedious to screen the desired transformants, until the discovery of CRISPR technology. Nowadays, industries such as biotech, pharmaceuticals, agriculture, chemical etc. are using highly engineered industrial strain. The implementation of CRISPR-Cas tools has revolutionized genome editing and mitigated the investment in the metabolic engineering programs required to generate highly engineered microbial cell factories[10].

Continuing the quest for editing microbes of industrial importance, the review provides information about "CRISPR edited microbes and their industrial potential" researched in last six months (2019) from the reputed papers around the globe.

Microorganisms

Bacteria: Bioproducts from the microbes are less reactive, show structural diversity and are easily degradable causing less pollution. Bacteria are perhaps the most prolific microbial producers of bioactive natural products, which are represented by their secondary metabolites[45]. These secondary metabolites are utilized by humans through commercial production according to the need. With the ease of CRISPR Cas mechanism, industrial strain development becomes handy for such industrial productions.

***E.coli*:** Studies suggest that fermentation of glucose and xylose from the agricultural residues can be utilised by *E.coli* in the production of biobutanol .In a study, a dual-operon-based synthetic pathway in the genome of *E. coli* MG1655 engineered to produce n-butanol using CRISPR/Cas9 technology[1]. The technology can also be used to suppress the expression of many DNA loci in the genome. Scientists constructed a CRISPRi-mediated multiplex repression system to silence transcription of several endogenous genes

to increase precursor availability in a heterologous isopentenol biosynthesis pathway[47].

The development of a CRISPR–Cas9 based method for iterative genome editing and metabolic engineering of *Escherichia coli* enables the authors to integrate the β-carotene synthetic pathway into the genome and to optimize the methylerythritol-phosphate (MEP) pathway and central metabolic pathways for β-carotene overproduction[27]. Studies prominently done on the weak and previously ignored end-joining mechanism in *E.coli* can be now used for efficient large-scale genetic engineering assisted by CRISPR/Cas9[15] which shows its significance in industrial microbiology.

Clostridium: The genus *Clostridium* is composed of bioproducers which are important for the industrial production of chemicals as well as pathogens which are a significant burden to the patients and on the health care industry[28]. Li et al[26] developed a genomic editing tool (pCBEclos) for the use in *C. beijerinckii* based on the fusion of cytidine deaminase (Apobec1), Cas9 ^{D10A}nickase and uracil DNA glycosylase inhibitor (UGI), the method through which *C. beijerinckii* can be easily altered to reach its maximum efficiency.

Various clostridia such as *Clostridium acetobutylicum, C. tyrobutyricum, C. carboxidivorans, C. ljungdahlii* and *C. cellulovorans* are researched and modified to enhance butanol production by earlier metabolic engineering methods and hence CRISPR technology seems to be very promising[2,7,8].

Bacillus and Lactobacillus: A PCR screening for 81 selected *Lactobacillus sakei* isolates was performed and it identified 25 (31%) isolates as CRISPR–Cas positive with hypervariable spacer content[42]. To achieve higher yields, many researchers have performed genetic modifications and metabolic regulations on industrially important Bacillus species. It was demonstrated that the mutant *Bacillus licheniformis* with deletions of the genes amyL and chiA (encoding amylase and chitinase, respectively) by CRISPR/Cas9 system effectively improved the alkaline protease yield by 24.8%[55].

CRISPR approach is used to observe an editing efficiency of 76% which allowed multiple, rapid rounds of in situ editing of the subtilisin E gene to incorporate a salt bridge triad present in the *Bacillus clausii* thermotolerant homolog, M-protease[37].

CRISPR interference serves as the basis for functional characterization of *B. methanolicus* physiology in which scientists predicted the functions of Spo0A in sporulation and biofilm formation, MtlD for mannitol catabolism and catalase in hydrogen peroxide dismutation[40]. Swiss and Italian hard cheeses are manufactured by the use of *Lactobacillus helveticus*, a lactic acid bacterium which is thermophillic in nature and studies conducted on 25 *Lactobacillus helveticus* genome showed the presence and genetic heterogeneity of CRISPR loci[39].

Streptomyces: Controlling the metabolic production in a microbe, there should be a controlled regulatory system which can be easily induced to enhance the industrial yields. To achieve this, researchers and microbiologist employed the modular design approach to build a high performance synthetic inducible regulatory system that displayed a large dynamic range and thus, well-suited for the modulation of secondary metabolite production in Streptomyces[20].

Presently certain reviews depict attempts to provide a comprehensive summary for stable genomic engineering and to outline recent advances in these strategies such as CRISPR/Cas9, which have successfully manipulated Streptomyces strains to improve their biotechnological properties and increase production of natural or new gene-manipulated biologically active compounds[21].

Moreover, a genome-reduced industrial *Streptomyces chassis* L321 was rationally constructed and showed to exhibit several emergent and excellent performances for heterologous expression of secondary metabolite like enhanced intracellular energy (ATP) and reducing power (NADPH/NADP+), improved productivity of protein and secondary metabolite, more dispersed mycelia, increased transformation efficiency, simplified metabolite profiles, increased genetic stability[4]. Other modified bacteria with the help of CRISPR Cas mechanism are given in table 1.

Yeast: Taking about the various industrial productions in non-conventional yeast, we already know that they have potential role in the formation of biofuels, food additives and high valued proteins but due to deficiency of proper genetic tools in them, it becomes quite cumbersome to modify its genome.

Table 1
Other Bacteria modified by CRISPR Cas mechanism.

Bacteria	Type	Uses
Streptococcus thermophilus	Type I and Type II CRISPR-Cas	Chromosomal targeting[6]
Corynebacterium glutamicum	CRISPRi	L-lysine production[35]
Pseudomonas aeruginosa	CRISPR/Cas9	Genetic manipulation[9]
Ralstoniaeutropha	Type I and Type II CRISPR-Cas	31% RSSC genome have CRISPR[11]
Brevibacterium	CRISPR/Cas9	Cheese production[24]

However, new studies state that precise and maker free genome editing with CRISPR-Cas9 have shown great potential in synthetic biology and industrial biotechnology[5].

Saccharomyces: In the study conducted, a novel genome shuffling method was developed for *S. cerevisiae* using CRISPRCas, which results in the creation of the thermotolerant mutant strain T8-292, which can grow well at 39 °C, showing cell viability in low pH and high ethanol concentration[30].

The study and research done by Jiangping et al stated that CRISPRi system was constructed in β-amyrin producing strain *S. cerevisiae*SGib to repress the competitive pathway for β-amyrin production.

A CRISPR-based gene editing strategy that allows the systematic and meticulous introduction of a natural occurring mutation in the *FDC1* gene of genetically complex industrial *S. cerevisiae* strains, *S. eubayanus* yeasts and interspecific hybrids was conducted resulting in the cisgenic POF^- variants which show great potential for industrial application and diversifying the current lager beer portfolio[29].

***Yarrowialipolytica*:** *Yarrowialipolytica* seems to be very promising future microbial cell factory for various bioindustries. In one of the research papers published, author's main goal was the creation of a simple auxotrophic marker to test the efficiency of GoldenMOCS-based CRISPR/Cas9 genome editing in wild-type isolates of *Y. lipolytica*[12].

By using CRISPR as a screening method for biotechnologically important mutants, scientists developed a methodology to quantify the cutting efficiency of each sgRNA in a genome-scale library and in doing so to improve screens in the biotechnologically important yeast *Yarrowia lipolytica*[44].

***Rhodosporidiumtoruloides*:** A CRISPR/Cas9 system for genome editing in *R. toruloides* based on a fusion 5S rRNA–tRNA promoter for guide RNA (gRNA) expression, capable of greater than 95% gene knockout for various genetic targets[41] was done so that it can be used as a future metabolic engineering strategy.

Transforming a Cas9 expression cassette harboring nourseothricin resistance and selecting transformants on antibiotic resulted in strains of *R. toruloides* exhibiting successful targeted disruption of the native *URA3* gene[33] increasing editing efficiency upto 50%. Other modified yeast with the help of CRISPR Cas mechanism is given in table 2.

Fungi

Filamentous Fungi: The filamentous fungus *Fusarium fujikuroi* is well-known for its production of natural plant growth hormones: a series of gibberellic acids (GAs) in which some GAs including GA1, GA3, GA4 and GA7, are biologically active and have been widely applied in agriculture[46].

The little efficiency of old genetic tools resists researchers to modify the fungus but the use of CRISPR technology has removed this limitation. Author described various approaches taken to assure expression of the components necessary for editing and described strategies used to achieve gene disruptions, gene replacements and precise editing[43] using CRISPR.

***Aspergillus niger*:** Tong et al[49] in their review summarized the impact of systems biology on the citric acid molecular regulatory mechanisms, the advances in metabolic engineering strategies for enhancing citric acid production and discussed the development and application of CRISPR/Cas9 systems for genome editing in *A. niger*. Using the CRISPR/Cas9-dependent base editor and inducing nonsense mutations via single base editing, scientist inactivated the uridine auxotroph gene *pyrG*and the pigment gene *fwnA* with an efficiency of 47.36%–100% in *A.niger*[16].

A study conducted showed a modified CRISPR/Cas9 system for *A. niger* highlighted in two aspects: (1) construction of a single and easy-to-use CRISPR/Cas9 tool plasmid derived from pAN7-1 which was widely used in filamentous fungi (2) redesign of the easy-to-switch "ribozyme–gRNA–ribozyme (RGR)" element in the tool plasmid[54].

Researchers demonstrated the method by targeting the phenotypic marker *albA* and validated it by targeting the *glaA* and *mst*Cloci after which 100% gene editing efficiency was observed[25].

***Trichoderma*:** Engineering of *T. reesei* by multiplexed CRISPR/Cas9 in combination with the use of our recently established synthetic expression system (SES) enabled accelerated construction of strains which produced high amounts of highly pure lipase B of *Candida antarctica*(calB)[38].

Table 2
Other Yeast modified by CRISPR Cas mechanism.

Yeast	Type	Uses
PseudozymaAntarctica	CRISPR/Cas9	Efficient gene-targeting method[22]
Kluyveromyces lactis	CRISPR/Cas9	Sequencing to identify two mutations in KlSEC59 gene[56]

Algae

Microalgae: Researchers have been curiously focusing on the efforts made so far to targeted genome engineering of microalgae, identified scopes about the hurdles related to construction and delivery of CRISPR–Cas components, algae transformation toolbox and outlined the future prospect toward developing the CRISPR platform for high-throughput genome-editing of microalgae[36].

In the study conducted, authors developed a highly efficient transgene-free targeted mutagenesis and single-stranded oligodeoxynucleotide (ssODN)-mediated knock-in in *E. gracilis* using Cas9 RNPs, which provides the first evidence that *E. gracilis* is a genome-editable organism[32].

Issam et al[17] reviewed the strategies and status of the use of CRISPR technology to improve the efficiency of Eukaryotic microalgae to produce biologically active compounds.

Cyanobacteria: In *Geitlerinema*sp.FC II, a filamentous cyanobacteria is characterized by the presence of multiple CRISPRCas (Clustered Regularly Interspaced Short Palindrome Repeats – CRISPR associated proteins) clusters, multiple variants of genes encoding photosystem reaction centres, biosynthetic gene clusters of alkane, polyketides and non-ribosomal peptides due to which it has an ecological advantage over other strains for biomass production in large scale cultivation system and hence, FC II may be deliberately used for production of biofuel and other industrially important metabolites[3].

Cyanobacterium, Anabaena sp. PCC 7120, a heterocyst-specific conditional gene repression system was successfully created by combining a cell type-specific gene induction system with CRISPRi technology[14] which increases its ethanol productivity and hence became suitable for biofuel productions. Other modified actinomycetes with the help of CRISPR Cas mechanism is given in table 3.

Conclusion

Looking forward to the future of industrial biotechnology, CRISPR edited microbes are going to take the first position in the queue of genetic modification techniques. The part of bacterial adaptive immunity which is used to harness the full potential of microbes is gaining more and more recognition worldwide. However, new emerging technologies will outnumber old ones, yet this technology has a potential to revolutionize the way we work with the microbe, not only in industrial but in every field of biological application considering all the ethical aspects.

Table 3
Other Microorganisms modified by CRISPR Cas mechanism

Actinomycetes	Type	Uses
Streptomyces coelicolor	CRISPR/Cas9	Precise gene replacements and reversibly control gene expression[48]
Streptomyces	Cluster assembly, cloning and expression, CRISPR/Cas9 technologies and chassis strain development	Reprogramming biosynthetic pathways[34]

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