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Synthetic chromosome - the first element for synthetic life

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ABSTRACT

Synthetic biology is a scientific area, that link together domains such as: biotechnology, microbiology, system biology, genetic engineering and bioinformatics. The main goal of synthetic biology is to constructing and designing artificial biological systems or re-design of existing biological systems for useful purposes - synthesizing for knowledge and synthesizing for products. The main structure on which synthetic biologists focus is the chromosome. Thanks to the development of modern genome editing techniques such as CRISPR/Cas9, or DNA assembly methods in recent years, there are more and more possibilities of creating synthetic chromosomes. The current achievements give hope for the construction of a fully synthetic organism into the future, as well as open up new possibilities related to the synthesis of new products. The following paper presents actual knowledge about synthetic chromosome including the topic of genome editing method and potential applications of synthetic biology.

***Keywords*:** synthetic biology, synthetic chromosome, CRISPR/Cas9, λ Red, DNA assembly

1. INTRODUCTION

After synthesizing urea from ammonium and hydrogen cyanide by Friedrich Wöhler in 1828, scientists are trying to recreate and improve capabilities of biological organisms. The culmination of this, is the emergence of synthetic biology. Synthetic biology is a scientific area, that link together domains such as: biotechnology, microbiology, system biology,

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genetic engineering and bioinformatics. Over the years, scientists have created many definitions of synthetic biology [1], [2]. The main goal of synthetic biology is to constructing and designing artificial biological systems or re-design of existing biological systems for useful purposes. The development of this field of science began about 20 years ago, with the publication in Nature, about genetic toggle switch, related to *Escherichia coli* genes [3]. With the evolution of molecular biology techniques, in the following years, the possibilities of creating artificial life increased.

The main structure on which synthetic biologists focus around the world is the chromosome - the form of the organization of genetic material in the cell. We have many reasons to create artificial chromosomes. In a very good way, Schindler and Waldminghaus summarized this in their publication in one sentence – "Synthesizing for knowledge and synthesizing for products" [4].

The following paper presents actual knowledge about synthetic chromosome including the topic of genome editing method and potential applications of synthetic biology (microbiology) in science and industry.

2. IDEAS FOR CREATING A SYNTHETIC CHROMOSOME

In this chapter we will discuss three ideas to creating synthetic chromosome. Some of them are already carried out in laboratories. Most of the methods are based on bacterial chromosomes. This is mainly due to the simplicity of bacterial genomes.

2. 1. Minimal chromosome

Table 1. Genome sizes of different bacteria together with semi-synthetic organisms (grey marking).

Species name	Number of genes	Size (kb)	Biotic relationship	Reference
Nasuia deltocephalinicola	137	112	symbiotic	[9]
Buchnera aphidicola Cc	431	420	symbiotic	[6]
JCVI-syn3.0	473	531	free living	[10]
Mycoplasma pneumoniae	736	816	free living	[6]
JCVI-syn1.0	901	1079	free living	[7]
Haemophilus influenzae	1774	1830	free living	[6]
Pseudomonas aeruginosa	5742	6264	free living	[6]

The most important reason why we should strive to create the smallest possible chromosome is low energy consumption during the genome assembly. In reaching the minimum genome, it is very important to determine the number of genes (essential genes) necessary to survive the organism. This distinction can be demonstrated through dependence - genes that do not contain transposons in their structure are likely to be essential genes [5].

To generate the smallest possible genome, most studies were based on bacteria, which naturally have a small number of genes [6]. The most known experiment related to the minimal genome was carried out at the Venter Institute in 2010. Scientists based on *Mycoplasma mycoides* created a semi-synthetic organism (JCVI-syn1.0) capable of self-replication, in which the genetic material was replaced by a synthetic chromosome created in the laboratory [7]. In 2016, the same team created another organism with only 473 genes - JCV-syn3.0 - it is a free living organism, with the smallest number of genes [8]. Table 1 shows the size of the genomes of various bacteria along with semi-synthetic organisms.

2. 2. Chromosome refactoring

Refactoring is a definition derived from computer science. It means the process of rebuilding the computer code without changing its main function, leading only to its simplification. In recent years, the idea of refactoring has been used to create biological systems [11], [12]. The groundbreaking work utilizing the refactoring process was based on the bacteriophage T7 [13].

Due to the complexity of the genetic code, its reconstruction would significantly facilitate its understanding and manipulation. The refactoring process can also be used to eliminate redundant genes, which is closely correlated with the creation of a minimal chromosome.

2. 3. More chromosomes better than one

Most bacteria in contrast to the eukaryotic organisms carry only one chromosome, apart from a few exceptions [14]. *Bacillus subtilis,* is a model organism, used to study the replication of a bacterial chromosome. Dividing the *B. subtilis* chromosome (4 146 kb) into eight smaller chromosomes would lead to replicons of approximately 520 kb. This size is similar to the JCVI-syn3.0 artificial chromosome [10]. Dividing the *B. subtilis* chromosome into thirty-seven smaller variants would give the size close to the *Nasuia deltocephalinicola* genome - the organism with the smallest known bacteria genome (112 kb) [9].

More chromosomes of smaller size than the original one will significantly facilitate the editing and rearrangement of genes. For example, a larger amount of chromosomes can be used to divide genes for function. The work of Liang and others [15] proves that it is possible, but still requires many improvements.

3. STATE-OF-THE-ART METHODS FOR SYNTHETIC CHROMOSOME

In the previous chapter, we presented the concepts of creating a synthetic chromosome. In the next one, we want to present techniques enabling its creation: genome editing and DNA assembly.

3. 1. Chromosome editing

One of the first methods used to edit genes was the λ Red method. Datsenko and Wanner demonstrated the effect of the Red recombination system on the recombination capacity between the *Escherichia coli* bacterial chromosome and the linear fragment of dsDNA. This system is based on three proteins (exonuclease λ, proteins β and γ) that activate the hyper-rec state in the cell (Fig. 1). The linear fragment is flanked by sequences homologous to the locus and introduced by transformation into a cell. After the system is activated, a single insertion occurs [16]. Despite its precision, due to the many variables, the efficiency in the point mutations is 0.1-10%. For larger mutations, this value drops to 10^{-4}-10^{-5} [17]. To increase the efficiency of the method, the Red system is combined with other methods, like CRISPR/Cas9.

Prokaryotic organisms exposed to the harmful effects of bacteriophages and exogenous genetic material have evolved something like the immune system - the CRISPR/Cas system. The system consists of two parts: CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and proteins from the Cas group. One of the versions - CRISPR/Cas9 - has found a wide application in genetic engineering . This method uses gRNA (guide RNA) to bind to the selected site in the genome, and the Cas9 endonuclease performs a precise DNA helix cut. The resulting gap is repaired by non-homogeneous joining of the ends (NHEJ) or homologous recombination, resulting in a deletion or insertion. Most genomic editions using CRISPR/Cas9 were performed on eukaryotic organisms [19], [20]. This is due to the many abnormalities during homologous DNA repair and NHEJ in bacteria and archaea, which often contributes to their death.

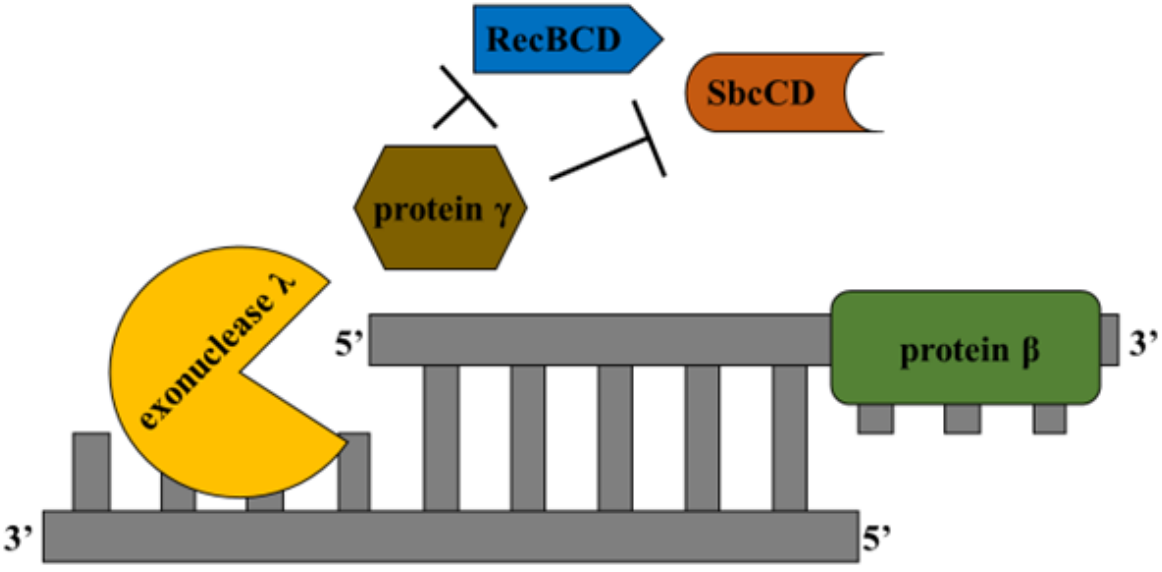

Figure 1. Components of the Red recombination system: exonuclease λ - forms sticky ends 5 '→ 3', protein β - promotes annealing to the complementary DNA fragment in the target cell, protein γ - protects DNA against bacterial endonucleases; based on [18].

Despite the low CRISPR performance, this method in combination with λ Red increases the efficiency and selectivity of gene editing. If a mutation does not occur with

λ Red method, then the appropriately programmed gRNA will recognize the unchanged sequence and the Cas9 nuclease will create double stranded breaks, that are lethal (Fig. 2). This system functions both in bacteria of high recombination (*Streptomyces sp.*) and in species with low recombination frequency (*Escherichia coli*) [21].

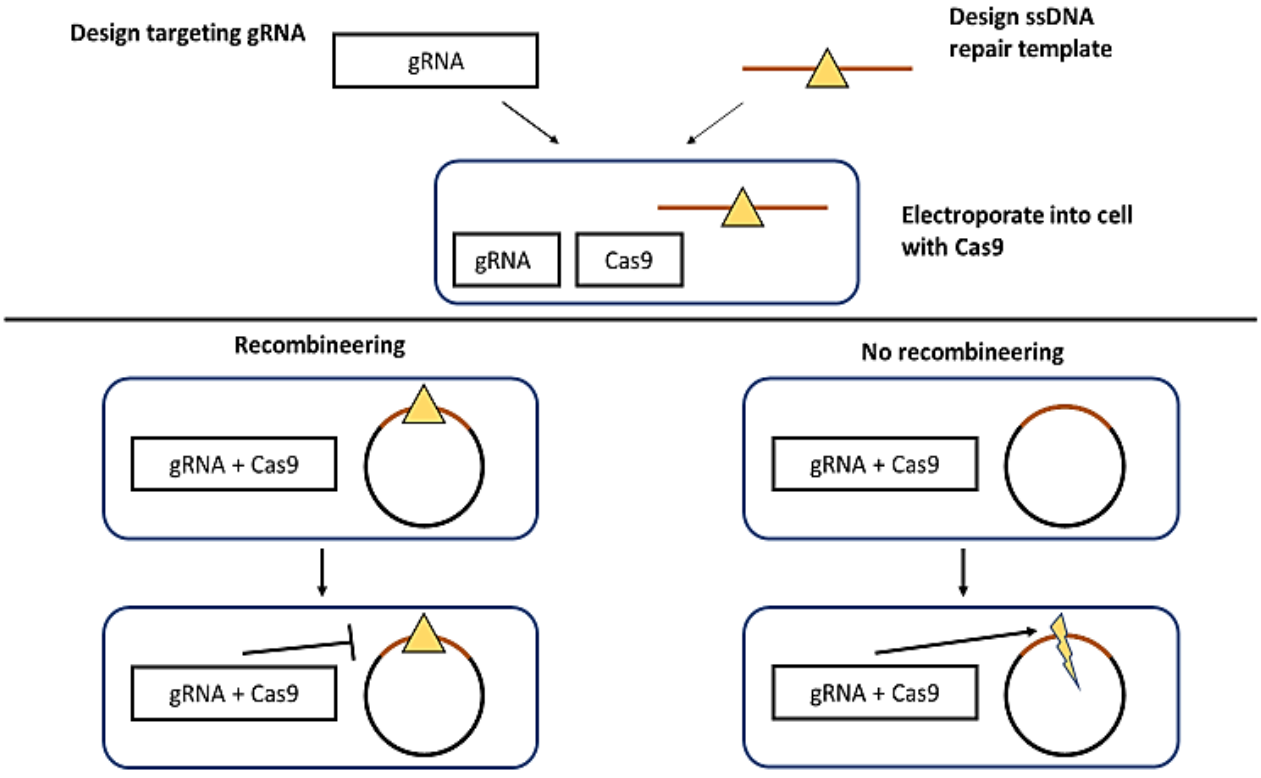

Figure 2. The mechanism of the CRISPR/Cas9 system related to the λ Red system.
(Modified from: https://blog.addgene.org/crispr-methods-for-bacterial-genome-engineering)

Prokaryotes, that can be efficiently genetically modified using these methods belong to the minority. To overcome this problem, the Lartigue *et al.* used *Sacharomyces cerevisae* cells to edit the genome of *Mycoplasma mycoides*. Bakery yeast is a well-known model organism on which numerous manipulations can be carried out. In this method, the bacterial genome is transferred to the yeast cell, then modified with the tools available for *Sacharomyces cerevisae* and transferred back to the bacterial cell [22].

3. 3. DNA assembly

In the past, a system based on endonucleases recognizing specific restriction sites and DNA ligase, was used to perform insertions. Unfortunately, this method does not work when assembling larger DNA fragments. Restrictions sites are very rare, they occur on average every 4 kb. For this reason, there are very few restriction sites on some chromosomes, and on some plasmids there is often only one such place. The solution to this problem may be a method based on DNA homology [4].

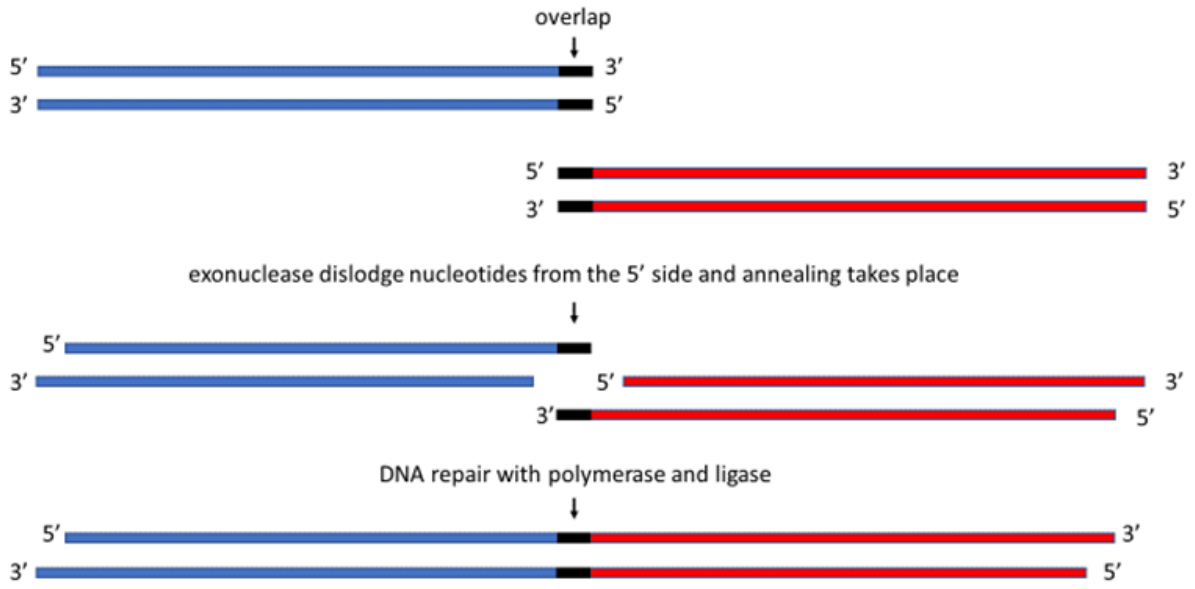

Figure 3. Gibson Assembly method; based on [7].

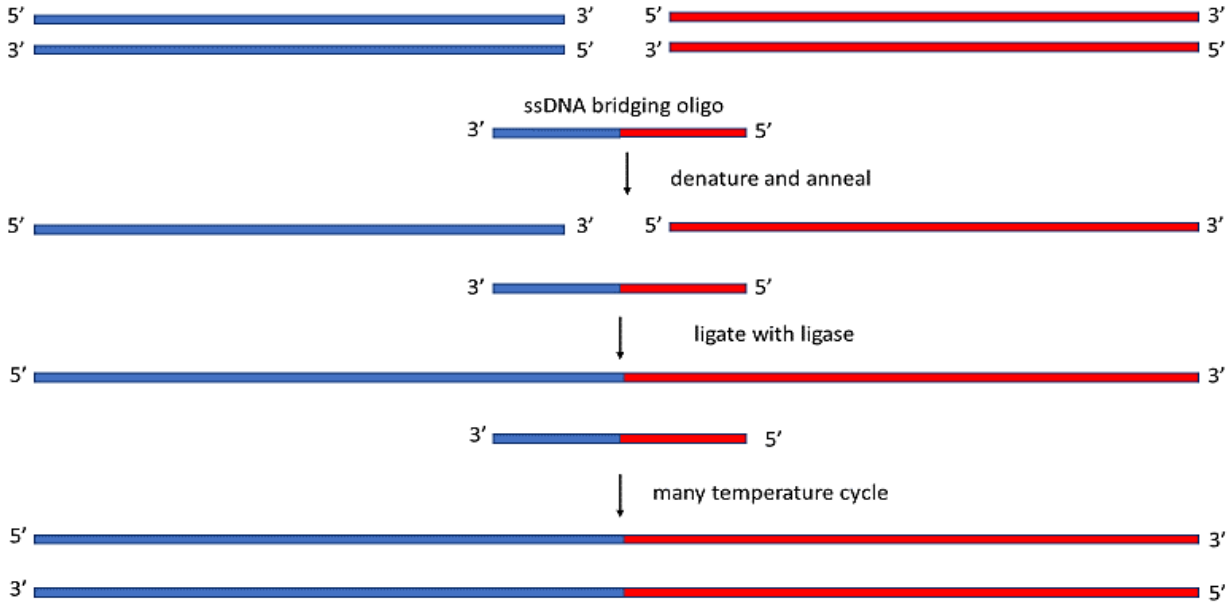

Figure 4. Assembly ligase cycling reaction method; based on [23].

The oldest method based on DNA homology was developed by Gibson, the founder of the first *Mycoplasma* with a synthetic chromosome. In this method, the DNA fragment must be overlapping at the ends with a 40 bp sequences. Together with the DNA sequence, the following enzymes are introduced into the cell: DNA polymerase, DNA ligase and 5 'exonuclease. Exonuclease creates matching free ends, which are effectively combined with each other. The resulting gap is supplemented by DNA polymerase and ligase (Fig. 3) [7].

The efficiency of the Gibson method for a larger number of assemblies drops significantly (< 50%). A more efficient alternative (60-100%) is the assembly ligase cycling reaction (LCR). This method is based on ssDNA bridging oligo. A designed oligonucleotide bridges two sequences that are complementary to the ends of neighbouring DNA sequences. After binding of both sequences with the thermostable ligase, there are many cycles: denaturation-annealing-ligation, designed to assemble the DNA constructs in an efficient manner (Fig. 4) [23]. This method is distinguished by the fact that it is highly efficient, fast and cheap.

4. SYNTHETIC ORGANISMS APPLICATION

Referring to the introduction of the publication, the usefulness of synthetic biology is considered in two categories: science knowledge and applications. Currently, new methods in synthetic biology are expected to have wide application. Especially in areas such as: biotechnology, medicine and industry. The possibility of interfering and designing the constituent parts of the genetic code allows us to create an organism that produces the desired substances. Until now, only several applications have been implemented using bacteria and an synthetic created chromosome. For this reason, the examples described below are based on the organisms of prokaryotes as well as on eukaryotic organisms.

Nowadays, genetic engineering tools are used in many areas (Fig. 5.), individual ones will be described in a larger scope.

4. 1. Environmental protection

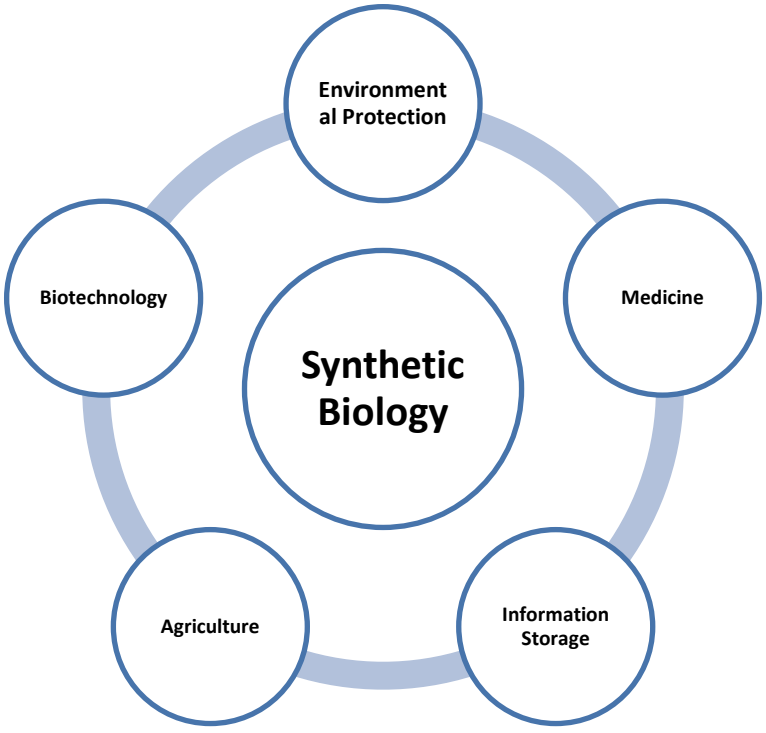

Figure 5. Application of synthetic biology in various fields of science.

The use of synthetic biology tools in ecology is extremely necessary. Intensive use of natural resources leads to their exhaustion. For this reason, we must look for new producers of biofuels among microorganisms.

Currently, the environment is the most polluted by the packaging of food, cosmetics and foil used in industry. To avoid this, it would be necessary to use packaging made of biomaterials. However, this is not easy, because the production of biomaterials must meet many requirements. Above all, the production process must be efficient and economically viable. However, the most important requirement is to create a microorganism that will produce biological substances for bio-packaging products.

New mutants of *Halomonas sp*. that are able to produce polyhydroxyalkanoates (PHA) have been successfully designed. Their biggest advantage is their easy biodegradation, thanks to which they are used to produce biopacks. By interfering with the genome of various bacteria, the yield of polyhydroxyalkanoates can be increased. The literature also reports on other bacterial strains, such as *Pseudomonas sp.*, which are also capable of producing PHA [24].

4. 2. Agriculture

In the agricultural sector, there is also a growing need to apply the latest synthetic biology technologies. One of the main challenges for modern agriculture is the constantly growing number of people in the world. Therefore, the aim is to increase the nutritional value of nutrients obtained from plants [25]. An example can be oil plants in which the improvement of the nutritional quality of oils can be improved by modifying genes. An experiment was carried out in which the model *Arabidopis thaliana* was used. Its genome has been modified using genes responsible for the production of seven different enzymes from five organisms, such as yeasts and algae [26].

An example of the use of bacteria with an synthetic chromosome are bacteria from the *Actimomyces* species that produce avermectins. Avermectins are antimicrobials used in the cultivation of plants [27]. They have an anthelmintics effect. Unfortunately, genetic manipulations on bacteria are expensive and time-consuming. Therefore, there is a small number of studies conducted on *Streptomyces avermitilis* regarding the production of new avermectin derivatives. The latest idea of scientists is to place newly constructed genes responsible for the production of avermectin in a synthetic bacterial cell. The result would be a reduced cost of avermectin production and time savings [28].

4. 3. Biotechnology

Baker's yeast as a model organism are widely used in scientific experiments. An extremely important feature contributing to the wide use of *Saccharomyces cerevisiae* in the study, is the ease of carrying out genetic manipulation and simple breeding. With increasing interest in synthetic biology, the base of available genetic engineering tools is also growing. Thanks to this, we can use *Saccharomyces cerevisiae* cells as specific factories. It can be seen that one of the more commonly used genetic engineering methods used for yeast cells is CRISPR/Cas9. The use of CRISPR/Cas9 and similar methods allows the design of yeast production of selected bioproducts [29]. For example, already about 10 years ago, we succeeded in obtaining the *Saccharomyces cerevisiae* strain, which carries out the biosynthesis of a commonly used flavoring agent, vanilin [30].

As often as eukaryotic organisms, bacteria are used in biology and synthetic research. Currently, innovative solutions in the diagnosis of diseases are intensively sought for. It aims to solve medical problems using synthetically constructed genetic systems. Bacteria have the ability to shift towards different signaling molecules. One of these signaling molecules is hydrogen peroxide, the secretion of which can be evidence of disease. This process is called chemotaxis. Therefore, there is an idea that bacteria would follow the source of hydrogen peroxide. The solution may be modern synthetic biology methods that will allow the genetic construction of *E. coli* as a bacterium locating the site of hydrogen peroxide evolution [31].

Table 2 shows selected applications of synthetic biology in various fields of science.

Table 2. Applications of synthetic biology in various fields of science.

Branch of science	Organisms	Effect of changes in the genome	Reference
Environmental protection	*Halomonas sp.*	Production of PHA	[24]
Agriculture	*Arabidopis thaliana*	Increased secretion of nutrients	[26]
Agriculture	*Streptomyces avermitilis*	Production of avermectin	[27]
Biotechnology	*Saccharomyces cerevisiae*	Biosynthesis of vanillin	[30]
Biotechnology	*Escherichia coli*	Construction of a bacterial disease diagnosis system	[31]

5. CONCLUSIONS

Thanks to the development of modern genome editing techniques such as CRISPR/Cas9, or methods of DNA assembly in recent years, there are more and more possibilities of creating synthetic chromosomes. The appearance of the semi-synthetic organism in 2010 significantly accelerated the development of synthetic biology. Although most of the research is carried out on a bacterial chromosome and is in the experimental phase, part of the acquired knowledge is used to edit the genomes of higher organisms. The current achievements give hope for the construction of a fully synthetic organism into the future, as well as open up new possibilities related to the synthesis of new products.

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