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Review Article

Evolutionary Engineering Applications in Microbial Ethanol Production

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Abstract

Microbial biofuel production using renewable resources is an important alternative to conventional petroleum-based fuels. In this respect, conversion of the cellulosic biomass as the renewable resource to simple sugars and biofuels is the main strategy. Among a variety of biofuel types, ethanol is a widely studied biofuel, and ethanol production from lignocellulosic biomass is a major field of research. For efficient ethanol production, improvements in both the producer microorganism and the process are required. Most of the research for the improvement of the microorganism focuses on sugar utilization, tolerance to inhibitor stresses that occur during ethanol production, and tolerance to ethanol as the product. As all of these properties are genetically complex (multigenic) properties, evolutionary engineering, based on random mutation and systematic selection of desired phenotypes without the need for prior genetic or biochemical information about the basis of the desired phenotype, is a powerful and practical strategy to obtain these desired phenotypes. In this review, evolutionary engineering applications of microbial ethanol production are discussed, regarding sugar utilization, inhibitor and ethanol stress tolerance.

ABBREVIATIONS

XR: Xylose Reductase; XDH: Xylitol Dehydrogenase; XI: Xylose Isomerase; PPP: Pentose Phosphate Pathway; NTG: N-Methyl-N'-nitro-N-nitrosoguanidine; 2-DG: 2-deoxy-d-glucose; SHCF: Separate Hydrolysis and Co-Fermentation; PMDS: Polydimethylsiloxane; HSSL: Hardwood Spent Sulfite Liquor; EMS: Ethyl Methanesulfonate

INTRODUCTION

Alternative fuel sources are becoming increasingly important, because of the scarcity and the increasing demand for fossil fuels, and the need for environmentally benign fuels [1]. Production of biofuels by microorganisms using renewable resources is an important alternative to petroleum-based fuels. The use of cellulosic biomass as a renewable resource by converting it into simple sugars and then to biofuels is the major approach. Metabolic engineering of microbial pathways for biofuel production includes a variety of biofuel types, such as alcohols, fatty acid alkyl esters, alkanes, and terpenes; as reviewed by Zhang et al. [2], based on studies with *Escherichia coli* and the yeast *Saccharomyces cerevisiae*.

Regarding alcohol production as biofuels, ethanol production from lignocellulosic material is a major research area. Recent studies have been focusing on metabolic engineering of industrial ethanol production hosts such as *S. cerevisiae*, *E. coli*, *Corynebacterium glutamicum* and *Zymomonas mobilis* for improved ethanol production.

Increasing microbial tolerance and conversion of inhibitory compounds found in lignocellulosic hydrolysates is another major area of research, regarding biofuel production. A variety of inhibitory compounds are formed during hydrolysis of lignocellulosic substrates. These inhibitors, such as furans, phenolics and weak acids, decrease ethanol yield and productivity. Additionally, at higher concentrations, ethanol as the fermentation product also has an inhibitory effect. Thus, to minimize inhibition and increase process efficiency and productivity, natural tolerance of *S. cerevisiae* has to be improved, and fermentation control strategies are necessary, as discussed previously [3]. Metabolic, genetic and evolutionary engineering strategies can be employed to improve tolerance of microorganisms against such inhibitors and to increase their productivity [3,4]. Among these strategies, evolutionary engineering is a powerful strategy to improve genetically complex microbial properties such as stress resistance. It is based on a systematic selection procedure that favors a desired phenotype [5,6]. The major advantage of this strategy is that it does not require any prior (genetic, biochemical, etc.) information on the desired phenotype. In addition, this approach can still be applied to improve industrial

organisms when rational metabolic engineering strategies are hindered by difficulties of cloning in industrial strains, mainly due to their genetic complexity such as ploidy or the lack of a genetic toolbox [7].

In this review, evolutionary engineering applications related to microbial ethanol production are considered. Firstly, evolutionary engineering studies for improved sugar utilization (such as xylose and mixed-sugar utilization) are described, as summarized in Table 1, along with the process improvements. Secondly, evolutionary engineering for improved tolerance to inhibitor stresses (such as hydrolysate, weak acids, furans) related to bioethanol production (Table 2) is discussed. Finally, evolutionary engineering studies for improved ethanol tolerance are considered, as summarized in Table 3. The molecular analyses to gain insight into the complex molecular basis of these properties are also mentioned in this review.

Xylose utilization

Xylose is the second most abundant sugar after glucose in lignocellulosic hydrolysates. Fermentation of both pentose and hexose sugars is desired to improve the economic feasibility of the lignocellulose-to-ethanol process. However, major ethanol-producing microorganisms cannot ferment xylose due to the lack of an endogenous xylose assimilation pathway and specific pentose transporters. To this end, *S. cerevisiae* and *Z. mobilis* have been engineered with heterologous xylose assimilation pathways. The heterologous xylose reductase (XR)/xylitol dehydrogenase (XDH) or xylose isomerase (XI) pathway has been expressed in fungi and bacteria to convert xylose to xylulose-5-P, which is further metabolized through the pentose phosphate pathway (PPP) and glycolysis [8].

Rational approaches to improve xylose fermentation focused mainly on the identification of more efficient enzymes for its transport and consumption [9], maintenance of the redox balance, the flux through the PPP and elimination of the by-product xylitol [8]. However, the ethanol yield and productivity of xylose fermentation is still not at comparable levels to that of glucose fermentation [8,10]. Rational strategies are hindered by the complexity of the regulation of xylose metabolism in the recombinant strains. However, combinatorial approaches using rational metabolic and evolutionary engineering are promising for efficient xylose conversion [11,12]. Recombinant xylose-fermenting strains have been further improved by laboratory evolution.

Xylose-utilizing *S. cerevisiae* strains have been evolved through serial batch cultivations, chemostat cultivations, or a two-stage process involving both modes of selection [13,14-16]. *S. cerevisiae* overexpressing *Piromyces* xylose isomerase (XYLA), *Pichia stipitis* xylulose kinase (*XYL3*) and the PPP genes has been subjected to adaptive evolution combining batch and chemostat cultivations to improve growth and xylose consumption rate. The evolved strain showed an upregulation of xylose isomerase due to the integration of multiple copies of *XYLA* in the chromosomes during the laboratory evolution process [16]. A combination of random strain improvement strategies can be applied to develop xylose-fermenting yeast strains. An industrial *S. cerevisiae* strain expressing xylose isomerase from *Clostridium phytofermentans*, enzymes of the pentose phosphate pathway and hexose transporter *HXT7* was subjected to a combinatorial strain improvement strategy, which involved EMS mutagenesis followed by genome shuffling and selection of xylose utilizing mutants on a xylose-enriched lignocellulosic hydrolysate. The mutants were further evolved through several rounds of evolutionary engineering in xylose medium [17]. The resultant variant showed increased xylose-consumption rate, which in turn produced 32% more ethanol than the reference strain during fermentation of Arundo hydrolysate.

Similar strategies employed with the yeast have been applied to recombinant *Z. mobilis* to improve xylose fermentation. A xylose-fermenting *Z. mobilis* strain was improved through adaptation over 80 days and 30 serial cultivations in a medium supplemented with xylose at a high concentration. The evolved

Table 1: Evolutionary engineering studies for improved sugar utilization.

Microorganism used	Evolutionary engineering strategy	Improvements achieved	Reference
S. cerevisiae	Aerobic and anaerobic sequential batch cultivation followed by growth in a xylose-limited chemostat	Higher xylose consumption rate	[16]
S. cerevisiae	Batch cultivation in the presence of gradually increased glucose-to-xylose ratio	Efficient xylose consumption in the presence of high glucose	[26]
S. cerevisiae	EMS mutagenesis followed by genome shuffling and selection on xylose-enriched lignocellulosic hydrolysate	Higher xylose consumption rate	[17]
S. cerevisiae	Batch culture selection under gradually decreased oxygen levels	Anaerobic xylose fermentation	[21]
S. cerevisiae	NTG mutagenesis and selection on galactose followed by serial batch transfer in the presence of xylose and 2-deoxy-d-glucose	Higher galactose consumption, relieved glucose repression	[30]
Z. mobilis	Serial dilution and subculture in the presence of xylose (5%)	Higher xylose consumption rate	[18]
Z. mobilis	Batch culture selection in the presence of 2-DG	Relieved glucose repression	[19]
E. coli	Serial batch culture in the presence of xylose (100 g/L)	Higher xylose consumption, relieved glucose repression	[27]

Abbreviations: EMS: Ethyl Methanesulfonate; NTG: N- Methyl-N'-nitro-N-nitrosoguanidine; 2-DG: 2-deoxyglucose

Table 2: Evolutionary engineering studies for resistance to stress related to bioethanol production.

Microorganism used	Evolutionary engineering strategy	Improvements achieved	Reference
Spathaspora passalidarum	UV mutagenesis followed by continuous selection in the presence of gradually increased acetic acid concentration in synthetic medium	Acetic acid tolerance	[40]
S. cerevisiae	Serial batch transfer in the presence of furfural	Furfural tolerance	[45]
S. cerevisiae	Serial batch transfer in the presence of gradually increased HMF	HMF tolerance	[48]
Scheffersomyces stipitis	Cultivation in a continuous fermentor in the presence of increasing concentrations of hardwood spent sulfite liquor	Inhibitor tolerance	[34]
S. cerevisiae	Cultivation in an anoxic chemostat using non-detoxified triticale straw hydrolysate supplemented with xylose	Inhibitor tolerance	[15]
S. cerevisiae	Serial batch transfers in the alternating presence and absence of acetic acid	Constitutive acetic acid tolerance	[42]
S. cerevisiae	UV-mutagenesis and direct selection followed by iterative cycles of genome shuffling	Tolerance to salt, sorbitol, peroxide, and acetic acid stress	[36]

Abbreviations: UV: Ultraviolet; HMF: 5-Hydroxymethylfurfural

Table 3: Evolutionary engineering studies for ethanol tolerance.

Microorganism used	Evolutionary engineering strategy	Reference
S. cerevisiae	Cultivation of EMS-mutagenized or non-mutagenized cells in chemostat in the presence of increasing ethanol concentration	[51,52]
S. cerevisiae	Serial batch transfer in the presence of gradually increased ethanol concentration	[53,54,58]
S. cerevisiae	Prolonged cultivation in a turbidostat with gradually increased ethanol levels	[57]

Abbreviations: EMS: Ethyl Methanesulfonate

strain could grow on 10% (w/v) xylose and able to convert xylose to ethanol within two days while yielding high ethanol [18]. Mohagheghi et al., used a non-hydrolysable glucose analog, which causes glucose repression, through a batch selection of xylose-fermenting *Z. mobilis* and obtained a strain that could use more than 50% of the xylose present in pretreated corn stover, while the parent strain did not utilize any xylose [19].

One of the drawbacks of evolutionary engineering is the long experimentation times required. Optimization of the adaptation process may enable success in evolutionary engineering in a shorter time frame. Data showed that the inoculum size and the growth phase of the inoculum at the time of serial transfer were important parameters for a rapid batch culture selection to obtain variants with higher growth rate on xylose. Low inoculum size (0.5 %) and serial transfer of the yeast cells at exponential phase enabled to obtain a variant with a 3.9-fold increase in xylose consumption rate in 24 days [20]. Batch culture selection under gradually decreased oxygen availability can be applied for improvement of xylose fermentation under anaerobic conditions [21].

Evolutionary engineering studies may help identify novel gene targets for rational metabolic engineering. The mutants with improved xylose utilization have been investigated through gene expression microarray analysis and whole-genome sequencing [22-24]. Transcriptional differences among the mutants obtained by evolutionary engineering of a recombinant *S. cerevisiae* and the reference strain suggested that upregulation of thiamine and S-adenosyl methionine biosynthesis and downregulation of genes involved in Fe(II) transport contributed to the improved xylose utilization by the evolved strain [22]. Genetic analysis of evolved strains showed that mutations in *ASK10*, which encodes a stress-regulator protein, lead to an improvement of xylose isomerase activity through upregulation of genes encoding molecular chaperones [24]. Similarly, genome sequencing of other evolved strains revealed Pho13p, an alkaline phosphatase, as a novel target for enhanced xylose utilization.

Mixed-sugar utilization

Inhibition of uptake and consumption of xylose in the presence of glucose has been observed in the recombinant *S. cerevisiae* strains. Similarly, high concentrations of glucose inhibit xylose metabolism in *E. coli* [12]. *S. cerevisiae* lacks specific pentose transporters, although it encodes a large number of hexose transporters with different substrate specificity and affinity. Pentoses cannot compete effectively with glucose for cellular uptake due to the higher affinity of transporters for the latter [25]. Evolutionary engineering of xylose-utilizing *E. coli* and *S. cerevisiae* releases carbon catabolite repression and enhances the resistance of xylose metabolism to glucose [12,14]. An evolution experiment based on chemostat cultivation of xylose-fermenting *S. cerevisiae* lacking main Hxt1-7 and Gal2 transporters revealed Hxt11, a cryptic low-affinity glucose transporter, as a novel genetic target for efficient xylose transport. Directed enzyme evolution of Hxt1 yielded variants at position N366, which had improved affinity for xylose and allowed a balanced transport of glucose and xylose [25]. Similarly, a xylose-utilizing *S. cerevisiae* with an impaired glucose uptake was evolved through batch cultivation. As an alternative selection strategy, the strain was evolved through batch cultivation on xylose and gradually increased concentrations of glucose. The xylose consumption rate of the evolved strain was increased and not impaired by the

presence of glucose. The phenotype was attributed to a mutation in *CYC8* gene, which is a general transcription co-repressor involved in sugar metabolism and cell wall biogenesis [26]. In addition, evolutionary engineering of *E. coli* revealed a single mutation in *xylR* that enabled up to four-fold increase in xylose utilization in *E. coli* strains when cultivated in a glucose-xylose mixture [27].

Heterologous pathway importation and evolutionary engineering approach have been expanded to the utilization of other pentoses by *S. cerevisiae* [8,28]. Both XR/XDH and XI-based xylose catabolic pathways were expressed in a recombinant arabinose-utilizing *S. cerevisiae* to achieve simultaneous conversion of the two pentoses and glucose. This recombinant strain was evolved through batch cultivations on xylose under oxygen-limited conditions,and the individual variants were selected on glucose and xylose plates. Total pentose fermentation was successfully improved without a decrease in glucose consumption [28].

Glucose and galactose are the most prevalent sugars in the hydrolysates of marine biomass. Galactose metabolism in *S. cerevisiae* is under glucose repression and essentially linked to respiration. *S. cerevisiae* strains that can effectively utilize galactose in the presence of glucose and under anaerobic conditions are desirable from the industrial point of view [29,30]. Mutant *S. cerevisiae* strains obtained by chemical (NTG) mutagenesis followed by direct selection on galactose plates were further evolved through serial batch cultivation in a medium containing xylose and 2-deoxy-d-glucose (2-DG). The evolved strain had moderately relieved glucose repression and, in turn, exhibited faster galactose utilization [30]. To enhance fermentative catabolism of galactose, Quarterman et al., constructed a respiration-deficient *S. cerevisiae* strain by deletion of *COX9* gene and employed evolutionary engineering in serial batch cultivation on galactose as the sole carbon source. This strategy yielded evolved strains with 2.5-fold increased galactose consumption rate and 4.8-fold volumetric ethanol productivity than the reference strain [29].

Process improvements involving evolutionary engineered strains

An evolutionary engineered xylose-utilizing *S. cerevisiae* strain (IBB10B05) was employed in a laboratory-scale separate hydrolysis and co-fermentation (SHCF) process. "On-site" production of hydrolytic enzymes was achieved by the cultivation of a *Trichoderma reesei* strain in wheat straw hydrolysate. Different configuration of process units was evaluated based on mass-balance analysis to identify parameters affecting the overall ethanol yield of the process. The study emphasized the importance of the effective conversion of the total sugars in the hydrolysate [31].

Co-utilization of sugars is hampered during the later stages of fermentation due to the accumulation of high concentrations of ethanol. When ethanol concentration reached higher levels, the xylose consumption ceased even after the diminishing of total glucose in the medium [21]. Zhang et al., developed a process that coupled fermentation and pervaporation technology for *in situ* removal of ethanol. An evolved xylose-utilizing *S. cerevisiae* strain was used in this process, and a significant increase in the total ethanol yield was achieved, owing to the extended sugar conversion [21]. Regarding the product inhibition, ethanol fermentation using membrane bioreactor technology is emerging as an alternative technology. In this system, membranes with high selectivity and ethanol flux are used for successful separation of ethanol. However, the closed-circulating operation causes adverse conditions due to the accumulation of metabolites other than ethanol and affects the performance of the strains. Ding et al., obtained an evolved *S. cerevisiae* with better performance in PMDS membrane reactor as a result of three successive fermentation experiments during 1530h of total operation time. The specific ethanol production rate was increased by about 20% compared to the reference strain [32].

Inhibitor stress

Lignocellulosic hydrolysates contain different types of inhibitors at varying amounts, depending on the raw material and the choice of the pretreatment method. The lignocellulose-derived inhibitors are mainly grouped as furan derivatives, weak acids, and phenolic compounds. The inhibitors reduce the biomass propagation and ethanol production. Therefore, the cellulosic ethanol production appeals for tolerant fermenting strains to these inhibitors [33]. Evolutionary engineering was successfully applied in strain improvement for inhibitor tolerance. Natural or recombinant xylose-utilizing yeast strains were improved by long-term cultivation in non-detoxified hydrolysates or the presence of selected inhibitors in synthetic media. The evolved strains gain the ability to maintain their physiological state under the inhibitor stress. Uncovering the mechanisms of inhibitor resistance conferred by evolutionary engineering enables the development of robust strains.

Tolerance to hydrolysate

Pereira et al., adapted *Scheffersomyces stipitis*, a naturally xylose-utilizing yeast, to hardwood spent sulfite liquor (HSSL), which is a xylose and inhibitor-rich by-product of acid sulfite pulping process. A stable inhibitor tolerant strain was obtained when the wild-type yeast was propagated for an extended time in a continuous reactor with increasing concentrations of non-detoxified eucalyptus HSSL [34]. Similarly, an industrial *C. glutamicum* strain was subjected to laboratory evolution under corn stover hydrolysate stress and a stable evolved strain with tolerance to the inhibitors in the hydrolysate was successfully obtained [35].

Evolutionary process can be enhanced by using mutagenized populations to start an adaptive evolution. A recombinant *S. cerevisiae* strain was subjected to long-term evolution, after chemical mutagenesis, in an anoxic chemostat using non-detoxified triticale straw hydrolysate supplemented with xylose. While the reference strain could not produce ethanol in the presence of inhibitors, the isolated strain was able to remove the inhibitor compounds present in the medium within 24 h and produced 1.54 g/L ethanol [15]. Pinel et al., used cross-mating-based genome shuffling as an alternative strategy to chemical mutagenesis to induce genetic variability prior to selection. First, mutant pools were generated by UV-mutagenesis and directly selected on undiluted HSSL. The tolerant mutants were further

subjected to five rounds of genome shuffling and selection in between each successive shuffling steps. The HSSL tolerant mutants had improved viability in the presence of salt, sorbitol, peroxide, and acetic acid stress [36]. The resistance levels of the evolved strains may differ in individual and combinations of inhibitors [37].

The comparative genetic analysis of evolved strains elucidated mutations and variation in copy number in *FLO* genes in a *S. cerevisiae* strain evolved for combined hydrolysate stress and heat stress. Further lipidomic analysis of the strains indicated the importance of peripheral lipids/membranes in the resistance to the combined stresses [38].

Weak acids

Lignocellulosic hydrolysates include a significant amount of weak acids, such as acetate formic acid and levulinic acid, resulting from the solubilization and hydrolysis of hemicellulose fraction. In particular, acetic acid is usually present at high concentration (1~10 g/L) in hydrolysates [39].

Spathaspora passalidarum with improved resistance to acetic acid was developed by using an evolutionary engineering strategy based on UV mutagenesis followed by continuous cultivation for 380 generations at increasing acetic acid levels in glucose- and xylose-supplemented synthetic medium. The ethanol productivity and yield in the presence of 4.5 g/L acetic acid was improved by 7-fold and 2-fold, respectively. When the acetic acid-tolerant strain was cultivated in inhibitor-rich *Eucalyptus globules* autohydrolysate, a significant sugar consumption without lag phase was observed [40]. Breeding between stress-resistant haploid strains is a promising approach to improve multiple-stress tolerance. A diploid industrial *S. cerevisiae* was continuously cultivated under acidic or temperature stress to develop an acid-tolerant and a high-temperature-tolerant strain. Further mating of the haploid strains obtained from each evolved strain yielded a diploid strain with resistance to both stresses simultaneously [41].

It was observed that the tolerance of the evolved strains selected by long-term cultivation in the presence of acetic acid was not constitutive and required a pre-exposure to acetic acid to be induced. Thus, an evolutionary engineering strategy involving serial transfers of alternating presence and absence of acetic acid was used for the selection of the constitutive acetic acid tolerance [42].

Furans

Furfural and 5-hydroxymethylfurfural are two significant furan aldehydes present in lignocellulosic hydrolysates. Inhibitory effects of furans on ethanol-producing microorganisms such as *Z. mobilis* and *C. glutamicum* have already been reported [43,44]. *S. cerevisiae* responds to furans through global metabolic changes during an extended lag phase, involving the maintenance energy levels, cofactor regeneration and recovery from cellular damage [45]. Evolutionary engineering enables selection for the shorter lag phase and rapid conversion of furfural and furan to their less toxic derivatives [46,47]. Evolutionary engineering strategies based both on long-term serial batch and chemostat cultivations were successfully used to obtain tolerance to furfural and 5-HMF [48,49].

Improvement of ethanol tolerance by evolutionary engineering

As ethanol has an inhibitory effect on yeast cells, yeast strains with high ethanol tolerance are highly desirable for efficient and high-yield bioethanol production processes. Thus, evolutionary engineering strategies have been employed to improve ethanol tolerance of *S. cerevisiae*. In our previous review [7], early studies to improve yeast ethanol tolerance have been mentioned. These include continuous selection in a chemostat culture for ethanol-tolerant *S. cerevisiae* [50], as well as the adaptive evolution of mutagenized and non-mutagenized *S. cerevisiae* in the presence of ethanol stress and transcriptomic analyses of the evolved mutant strains [51,52]. Similarly, Dinh et al., also employed repetitive cultivations with a gradual increase in ethanol in culture media [53,54]. More recently, it has been shown that thermotolerant *S. cerevisiae* strains obtained by laboratory evolution had improved tolerance to high concentrations of glucose and ethanol. However, they showed a 'trade-off' in growth at temperatures below 34°C [55]. Such trade-off situations in evolutionary engineering are commonly observed where some traits of an evolved strain may be reduced in their function, as the cost of the improved or evolved traits. Thus, a detailed physiological characterization of the evolved strains is crucial for strains improved by evolutionary engineering [7].

Comparative biochemical and gene expression (qRT-PCR) analyses of parental and ethanol-adapted *S. cerevisiae* obtained by evolutionary engineering revealed that cell membrane compositions were different in ethanol-adapted strains. Additionally, the expression levels of genes involved in fatty acid metabolism were higher in the mutant strains than in the parental strain [56].

It is also important to note that evolutionary engineering for ethanol tolerance may also result in ploidy changes or diploidization. In a two-year evolution experiment using a turbidostat, ethanol levels in the bioreactor were increased from 6 to 12% (v/v) in the bioreactor. It was also observed that as the yeast cells gained ethanol tolerance (up to 11% (v/v) ethanol), they also changed into the diploid state from either haploid or tetraploid ancestral cells [57]. Similarly, evolutionary engineering of haploid *S. cerevisiae* for ethanol tolerance under repeated batch cultivation conditions with gradually increasing ethanol stress levels also revealed ethanol-tolerant and diploid strains. These strains could tolerate up to 12% (v/v) ethanol and had significantly higher ethanol productivity and viability under aerated fed-batch cultivation conditions. Comparative transcriptomic and proteomic analyses of the ethanol-tolerant strains showed an increase in their glycolytic and ribosomal proteins and a decrease in their respiratory capacity [58]. The complex molecular basis of ethanol tolerance and the relationship between diploidization and ethanol tolerance is yet to be further investigated in detail.

DISCUSSION AND CONCLUSION

As an important biofuel, ethanol is highly demanded. Thus, ethanol production processes need to be continuously improved. These improvements include both the process and the ethanol-producing microorganisms, most commonly the yeast *S.*

cerevisiae. In this respect, evolutionary engineering is a powerful strategy for improving genetically complex microbial properties related to ethanol production, such as sugar utilization, inhibitor resistance, and ethanol tolerance. The 'omic' level comparative analyses of the evolved strains help us understand the complex molecular basis of those desired properties and enable further industrial improvements.

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