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MINI-REVIEW

Novel mutagenesis and screening technologies for food microorganisms: advances and prospects

Qinghui Yu[1,2] · Yuncheng Li[1] · Bo Wu[2] · Wei Hu[3] · Mingxiong He[2] · Guoquan Hu[2]

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Abstract

Microorganisms are indispensable in the food industry, but wild-type strains hardly meet the current industrial demands due to several undesirable traits. Therefore, microbial strain improvement offers a critical solution to enhance the food industry. Traditional techniques for food microbial improvement, such as the use of chemical mutagens and manual isolation/purification, are inefficient, time-consuming, and laborious, restricting further progress in the area of food fermentation. In this review, the applications of novel mutagenesis and screening technologies used for the improvement of food microbes were summarized, including random mutagenesis based on physical irradiation, microbial screening facilitated by a microtiter plate, fluorescence-activated cell or droplet sorting, and microscaled fermentation in a microtiter plate or microbioreactor. In comparison with conventional methods, these new tools have the potential in accelerating microbial strain improvement and their combined applications could create a new trend for strain development. However, several problems that could affect its potential application may include the following: the lack of specific mutagenesis devices and biosensing systems, the insufficient improvement of the mixed culture system, the low efficiency when using filamentous fungi and flocculating bacteria, and the insufficient safety assessment on harnessing genome-editing technology. Therefore, future works on strain improvement remain challenging for the food industry.

Keywords Strain development · Food microorganism · Mutagenesis · Screening · Microscale cultivation

Introduction

Microorganisms are indispensable in the food industry since they are suppliers of food and drinks, additives, and preservatives. They also serve as "chefs" conferring attractive food flavors (Kum et al. 2015) and aroma (Ardo 2006) that are essential in the culinary industry. In addition, the use of probiotics as a food supplement demonstrates notable health improvement by boosting the growth of human gastrointestinal microflora (Pandey et al. 2015). Altogether, these contributions imply that the food industry heavily relies on microorganisms and microbial biotechnology.

Wild-type (WT) microbial strains can hardly meet industrial demands because of their undesired traits such as low yield, low tolerance, low stability, or abundant by-products in some cases. Researchers have developed various techniques, such as genetic engineering, cellular fusion/hybridization, or adaptive evolution to do strain improvement. However, the applications of genetic modification tools including transposon mutagenesis, staggered extension, mining of novel genes, and random chimeragenesis are restricted in food microorganisms and in the food industry because of safety and risk concerns (Félix et al. 2019; Karabín et al. 2018). Food safety on genetically modified organisms (GMOs) remains a controversial issue among policymakers and consumers (Karabín et al. 2018; von Wright and Bruce 2003). What's more, the genetic, even cellular and

Qinghui Yu and Yuncheng Li contributed equally to this work.

✉ Bo Wu
wubo@caas.cn

✉ Wei Hu
hudongli007@impcas.ac.cn

[1] College of Pharmacy and Biological Engineering, Chengdu University, 2025 Chengluo Avenue, Chengdu 610106, China

[2] Biomass Energy Technology Research Centre, Biogas Institute of Ministry of Agriculture and Rural Affairs, 4-13 Renmin Rd. South, Chengdu 610041, China

[3] Institute of Modern Physics, Chinese Academy of Sciences, 509 Nanchang Rd., Lanzhou 730000, China

physiological information of most food microbial strains is different from that of lab strains. Thus, it is not suitable to employ techniques that are based on genetic manipulation for food microbe breeding. Besides, although cellular fusion/hybridization and adaptive laboratory evolution techniques are efficient (Li et al. 2017; Cao et al. 2012), they are laborious and time-consuming.

Of all the existing techniques designed for strain improvement, the use of random mutagenesis demonstrates notable advantages over the other technologies for the improvement of food microorganisms. First, random mutagenesis is independent of the host cell's genetic information making it efficient for microbes with unknown genomic backgrounds; thus, it is suitable for most of the food microorganisms. Second, this tool allows the generation of a mutant library with high genetic diversity, and its manipulation is relatively simple and cost-effective. Finally, it is not involved to controversial GMO issue. Altogether, random mutagenesis represents one of the most useful tools for developing and improving food microorganisms.

A critical step involved in mutagenesis is to select and evaluate desired mutants from the mutagenic library. However, conventional mutant screening is still heavily dependent on shake-flask culturing or manual selection in the food industry (Aleem et al. 2018; Spadiut et al. 2010), which is tedious and inefficient. It becomes far more difficult recently with the emergence of novel mutagenesis technologies that are highly efficient. In addition, assessment of selected mutants and optimization of bioprocessing for industrial scale represent a great challenge. Although flask-culturing remains dominating, this system only offers the "end-point" data and can hardly provide reproducible and reliable parameters that are crucial for the scale-up. Moreover, a scale-up system that relies on a large amount of culture media and expensive nutrient requirements would exacerbate the cost of production affecting downstream prices.

Recently, novel technologies for mutagenesis, screening, and microscale cultivation have accelerated the strain development of industrial microbes (Fig. 1). In contrast, the strain improvement in the food industry still relies heavily on conventional techniques, holding back its potential development. In this review, the recent advances on new mutagenesis and techniques for food microbial screening and cultivation are summarized, and further prospects and trends in strain improvement are discussed. The aim of this review is to address the importance of these novel techniques and to accelerate the food microorganism development.

Novel mutagenesis technologies

Chemical mutagens, ultraviolet (UV) irradiation, X-/γ-rays irradiation, and ^{60}Co radiation are the common techniques used to facilitate conventional mutagenesis. Although chemical and UV-mediated mutagenes are prevalent in the food industry, they pose risk to the health of operators (Table 1). Moreover, they are laborious and consume too many media. In this review, only the novel techniques promising in strain improvement in food industry are introduced.

Atmospheric and room temperature plasma-mediated mutagenesis

The demand for mutagenesis techniques that are highly efficient and safe for human health is increasing in the food industry (Table 1). One of them is the atmospheric and room temperature plasma (ARTP)-intermediated mutagenesis, which was developed through collaboration between teams of researchers from Tsinghua University in China (Zhang et al. 2014). Based on the principle of atmospheric pressure radiofrequency glow discharge, the high energy released during the formation of plasma gives rise to DNA mutations (Zhang et al. 2015c). In comparison with conventional methods, ARTP mutagenesis is user-friendly, safe, and fast, thereby generating higher mutation rate under room temperature (RT) conditions that are desirable for microbial growth. Such advantages allow ARTP mutagenesis to be applied widely to improve the traits of numerous microbial species (Li et al. 2015; Tan et al. 2015; Wang et al. 2014; Zhang et al. 2018a).

ARTP mutagenesis exhibits high efficiency in boosting food production as evident in several studies that show its successful application in developing strains of food microorganism (Table 2). Recently, ARTP mutagenesis has demonstrated its power in the overproduction of organic acid and/or fatty acid. In ARTP-mutated *Mortierella alpina*, intracellular arachidonic acid (ARA) can increase by nearly 2-folds and accounts for a relative increase by 6.65% from the total fatty acids (Li et al. 2015). In ARTP-treated *Yarrowia lipolytica* strains, the titers of α-ketoglutaric acid showed an increase by 51.8 and 45.4% in a 500-mL flask and 3-L reactor, respectively, when compared to the titers produced by the parental WT strain (Zeng et al. 2015). In *Bacillus coagulans*, the production of L-lactic acid by ARTP mutants increases substantially by over 40% in 5-L bioreactors (Lv et al. 2016). Microbes are also important suppliers of docosahexaenoic acid (DHA) whereby its health and clinical benefits trigger an increasing demand in the food and health industries. Recently, an ARTP mutant strain of *Schizochytrium* sp. can produce 14.0 g/L DHA after optimizing the Fe^{2+} supplementation in shake-flask culture. Its lipid and DHA contents are 31 and 26% higher than the wild-type *Schizochytrium*, respectively (Zhao et al. 2018). In acetic acid bacteria (AAB) *Acetobacter pasteurianu*, ARTP treatment does not only lead to an enhanced ethanol tolerance by reducing membrane permeability but also boost significantly the acetic acid titer by nearly 4-

folds (Wu et al. 2015). In addition, ARTP mutagenesis also enables high production of amino acids (Cheng et al. 2015; Wang et al. 2015; Zhang et al. 2018b), vitamins (Cai et al. 2018; Xu and Zhang 2017), terpenoids (Qiang et al. 2014; Zhang et al. 2016), polyols (Liu et al. 2017b), enzymes (Jiang et al. 2017; Zhu et al. 2017), polysaccharide (Song et al. 2018), aroma (Wang et al. 2018), and additives (Lin et al. 2016) intended for the food industry (Table 2).

Aside from the enhanced production ability of ARTP mutant strains, the technique can also create mutants that reduce a considerable amount of detrimental by-products, such as the carcinogenic substance ethyl carbamate (EC) from fermented food and urea from fermented beverages. In the soy sauce fermentation, an ARTP mutant strain of *Bacillus amyloliquefaciens* does not only exhibit enhanced arginine production and salinity tolerance but also can reduce the level of EC and citrulline (an EC precursor) by 19.3 and 15.6%, respectively (Zhang et al. 2017). The same study also suggests that ARTP shows stronger mutagenic effect compared to UV irradiation. In the brewing industry, ARTP mutagenesis could suppress the undesirable metabolites produced by the wild-type *Saccharomyces cerevisiae* during fermentation. An industrial ARTP mutant *S. cerevisiae* reduces urea level by 50.6% in rice wine. Such reduction is likely associated with the upregulation of *DUR1*, *DUR2*, and *DUR3* genes (Cheng et al. 2017). In addition, ARTP technology also contributes to the reduction of toxic methanol (Liang et al. 2014) or acetaldehyde (Liu et al. 2018) in brewing yeast. As shown in Table 2, the improvements of multiple traits occur frequently

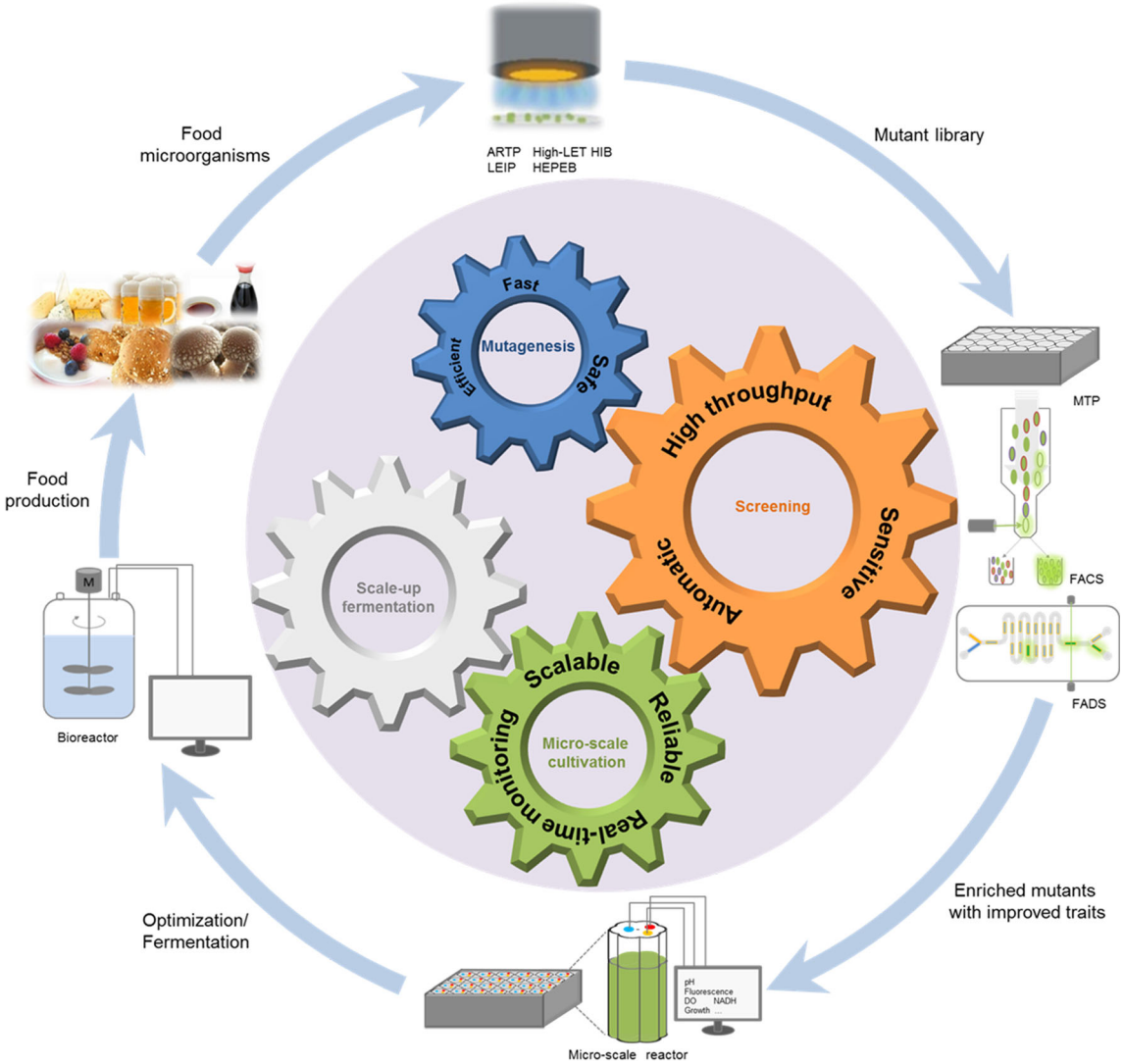

Fig. 1 The application of novel technologies for microbial strain improvement in the food industry. The mutagenic library can be created by fast, safe, and efficient mutagenesis technologies, including ARTP, high-LET HIB, LEIP, HEPEB, etc. The mutants with the desired trait are selected/enriched via sensitive, automatic, and high-throughput screening technologies. Then the enriched mutants are subject to micro-scale cultivation coupling with online monitoring, acquiring scalable and reliable bioprocessing parameters. These optimized parameters enable the scale-up fermentation

Table 1 Comparison of mutagenesis techniques

"Mutagen"	ARTP	LET-HIB	HEPE	LEIP	LIR	UV/chemicals
Operating safety	High	Unknown	Unknown	Unknown	Unknown	Low or toxic
Operation simplicity	Simple	Complicated	Complicated	Complicated	Complicated	Simple
Mutation efficiency	High	High	High	High	High	In variety, low to medium
Mutational spectrum	Wide	Wide	Wide	Wide	Unknown	Limited
Penetrating ability to liquid cultures	Weak	Strong	Weak	Weak	Unknown	Weak
Equipment/system size	Small	Large	Large	Large	Unknown	Small
Operating cost	Mediate	High	High	High	High	Low
Commercial availability	Yes	No	No	No	No	Yes

ARTP atmospheric and room temperature plasma, *LET-HIB* high linear energy transfer heavy ion beam, *HEPE* high-energy pulse electron beam, *LEIP* low-energy ion implantation, *LIR* laser irradiation

by using ARTP mutagenesis, suggesting that this tool is efficient and powerful in mutating food microbes.

Iterative ARTP mutagenesis is also a powerful strategy in improving multiple properties. A recent report on the ethanologenic bacterium *Zymomonas mobilis* shows that a multiplex mutagenesis strategy could create mutants with enhanced acetic acid and low pH tolerance (Wu et al. 2019). It is also demonstrated as a feasible way in food microorganisms. For example, the *Sporolactobacillus* sp. mutant YBS1-5 (Table 2) obtained from two rounds of ARTP treatment shows further improvement as compared to the mutant from the first ARTP treatment (Sun et al. 2015).

High linear energy transfer heavy ion beam-mediated mutagenesis

Heavy ion beams (HIBs) can generate high linear energy transfer (LET) that induces an increased proportion of DNA double-strand break causing large DNA deletions and/or rearrangements (Hu et al. 2017b; Kazama et al. 2007). Compared to low LET irradiation such as UV rays or X-/γ-rays, high LET-HIB-mediated mutagenesis displays a wider mutation spectrum and a higher mutation frequency, thus emerging as an efficient breeding method (Kazama et al. 2008).

While the continuous use of high LET-HIB for breeding is usually employed in other organisms, its application to food microorganisms is very limited with a few reports focusing mainly on the enhancement of production (Table 3). Using the fungus *Aspergillus niger*, Hu et al. (2016, 2017a, 2014) successively created some mutants that are citric acid overproducers via carbon ion irradiations. In particular, a mutant strain H4002 could boost the citric acid concentration up to 196.0 g/L with a production level of 3.3 g/L/h, which is currently the highest recorded quantity (Table 3). The same group also improve the L(+)-lactic acid production of *Lactobacillus thermophiles* by the HIB method. The HIB mutant SRZ50 shows enhanced lactic acid production using either glucose or fructose as a sole carbon source (Hu et al. 2018). Subclone of the bacteria obtained by another HIB treatment further shows substantial improvement in lactic acid production (Jiang et al. 2018).

Breeding of edible mushrooms underlies a great challenge, as it needs the right cultivation method for proper growth and fruiting body production. Recently, the edible mushroom *Tricholoma matsutake* with improved property has been obtained after gron-ion beams treatment with the LET of 310 keV/μm (Murata et al. 2018). The resulting mutant exhibits not only a dramatic change of phenotype but also shows enhanced capability of degrading dye-linked water-insoluble amylase and cellulose substrates. High LET-HIBs also demonstrate high efficiency in enhancing the production of DHA (Cheng et al. 2016), lipid (Wang et al. 2009a), and cellulase activity (Jiang et al. 2016a) in other genera.

Low-energy ion implantation-mediated mutagenesis

Energetic ions are obtainable under vacuum conditions by acceleration and mass selection. Hypothetically, mutagenesis by low-energy ion implantation (LEIP) is due to the combination of energy absorption, mass deposition, and charge transfer of energetic ions in cells (Gu et al. 2008). In comparison with conventional UV and X-/γ-rays and chemical mutagenesis, LEIP-mediated mutagenesis enables higher mutation rate and wider mutation spectrum (Table 1).

LEIP is a common technique used in plant breeding for decades, but its application in breeding food microbe begins just very recently. So far, its reported applications focus mainly on enhancing enzymatic activities and producing organic/fatty acids (Table 4). Proteases mediate the degradation of proteins into amino acids and small peptides, which contribute greatly to the specific flavor of fermented foods. The LEIP-mediated irradiation of the soy sauce producer *Aspergillus oryzae* results in significant enhancement of acidic and neutral protease activities during koji fermentation. Furthermore, the mutant *A. oryzae* exhibits substantial enhancement in protease secretion, protease mRNA expression, and mycelial

Table 2 Recent applications of ARTP mutagenesis for microbial strain improvement in the food industry

Mutant strain(s)	Improvement	Reference
For organic acids		
Yarrowia lipolytica 1-C6	Titers of α-ketoglutaric acid were enhanced by 51.8 and 45.4% in 500-mL shake flask and 3-L reactor, respectively	Zeng et al. (2015)
Mortierella alpina D20	Maximum arachidonic acid (ARA) production was 6.82 g/L, a 1.9-fold increase of intracellular ARA. The relative ARA content increased by 6.65% of total fatty acids	Li et al. (2015)
Acetobacter pasteurianus U1-1	Enhanced ethanol tolerance (11%) and decreased membrane permeability; titer of acetic acid reached 32.83 g/L or increased by 385.7%	Wu et al. (2015)
Aspergillus niger AA120	Enhanced tolerance against 20 g/L tannin; enhanced biomass to 32.9 g/L, 43.76% higher than the parent strain; boosted citric acid titer by 20.34% or 130.8 g/L	Zhang et al. (2018a)
Bacillus coagulans GKN316	45.39 g/L lactic acid was produced in the fermentation using a high concentration H_2SO_4 catalyzed steam-exploded hydrolysate	Jiang et al. (2016b)
Bacillus coagulans IH6 and IIIB5	L-lactic acid production was increased by 42.75 and 46.1% in 5-L reactors, respectively	Lv et al. (2016)
Aspergillus niger II-2-A1, IV-7-C6, and V-11-C5	Gluconate productions were 15.5, 32.8, and 12.1% higher than that of the parental strain, sugar consumption rates were 17.5, 17.3, and 30.6% higher than that of the parent strain	Shi et al. (2015)
Schizochytrium sp. mz-17	Maximum DHA titer was 14.0 g/L in 250-mL shake flask, over 2.1-folds higher than the wild-type; DHA percentage accounted for 50.9% of total fatty acid, nearly 2.2-folds higher than the wild type	Zhao et al. (2018)
For amino acids		
Corynebacterium glutamicum ARG3-15	L(+)-arginine titer is 45.36 g/L and productivity was 0.571 g/L/h, 50.8 and 66.0% higher than the starting strain; production of other extracellular amino acids in the mutant was reduced but L-arginine was increased	Cheng et al. (2015)
Corynebacterium glutamicum SYPS-062-33a	Mutant A36-pDser accumulated 34.78 g/l L(+)-serine with a yield at 0.35 g/g sucrose	Zhang et al. (2018b)
Streptomyces sp. FEEL-1	Enhanced the ε-poly-L(+)-lysine (ε-PL) productivity; ε-PL production was 68.1% higher than the parent strain	Wang et al. (2015)
For vitamins		
Bacillus amyloliquefaciens H.β.D.R.-5	Production of menaquinone-7 was increased to 52.6 mg/L in 500-mL flask and 61.3 mg/L in 7-L bioreactor using maize meal hydrolysate as feedstock	Xu and Zhang (2017)
Sinorhizobium meliloti 320	Improvement of vitamin B_{12} mutant MC5-2 reached 156 ± 4.2 mg/L production	Cai et al. (2018)
For terpenoids/lipids		
Blakeslea trispora	Improve the fermentation efficiency of lycopene by 55% concentration increase	Qiang et al. (2014)
Streptomyces movaraensi	Increase the production of glutamine aminotransferase by 27%	Jiang et al. (2017)
*Aspergillus niger*III-F-2, VII-F-6, IV-D-1	2.0–2.2 × 10^3 U/mL, ~ 70% higher yield of glucoamylase than the parent strain	Zhu et al. (2017)
Rhodosporidium toruloides np11	Improve the production of carotenoids and lipids; accumulated 0.23 g lipids/g cell dry weight and 0.75 mg carotenoids/g CDW	Zhang et al. (2016)
For reducing by-products		
Bacillus amyloliquefaciens ARTPC12	High tolerance to NaCl (18%); improved genetic stability; arginine titer was enhanced by 18.0%; citrulline concentration was reduced by 15.6%; EC concentration was reduced to < 13 μg/kg, a reduction of 19.3%	Zhang et al. (2017)
Saccharomyces cerevisiae S12	Methanol titer was reduced to 104.8 mg/L, 72.5% decreased methanol productivity; alcoholic content was increased to 15.3% (v/v), 8.9% higher than the starting strain	Liang et al. (2014)
Saccharomyces cerevisiae 5-11C	Expression of genes *DUR1*, *DUR2*, and *DUR3* in the mutant was reduced by 3.3- and 2.2-folds; urea content was reduced to 9–9.7 mg/L, 50.6% decrease in rice wine	Cheng et al. (2017)
Saccharomyces cerevisiae LAL-8a	Acetaldehyde content was reduced to 2.2 mg/L, 88.2% less than the parent strain	Liu et al. (2018)
Others		
Gluconobacter oxydans A-2-64	1,3-Dihydroxyacetone productivity was increased to 90.2 g/L in a flask culture, showing 26.3% higher than the starting strain	Lin et al. (2016)
Bacillus amyloliquefaciens FMME088	Enhanced the production of the acetoin; mutant H-5 produced acetoin up to 68.2 g/L in a shake flask	Wang et al. (2018)
Yarrowia lipolytica SWJ-1b	Mutant M53 produced 64.8 g/L erythritol from 100 g/L glycerol	Liu et al. (2017b)

Table 2 (continued)

Mutant strain(s)	Improvement	Reference
Hericium erinaceus 414, 236, and 323	Biomass was increased from 14.1 to 17.6%; mycelium polysaccharide content was increased ranging from 29.4 to 97.9%	Song et al. (2018)

morphology (Zhao et al. 2012). Aside from proteases, LEIP mutagenesis is also applied successfully to enhance the activity of xylanase (Li et al. 2007), thermostable α-amylase (Li et al. 2011), chitosanase (Su et al. 2006), and lipase (Ji et al. 2008). Moreover, LEIP mutant microbe enhances the production of lactic acid (Li et al. 2012b; Wang et al. 2009b), DHA (Fu et al. 2016), 1,3-dihydroxyacetone (Lin et al. 2016), sophorolipids (Li et al. 2012a), astaxanthin (Liu et al. 2008), and glutathione (Qian et al. 2013). These applications demonstrate the strength of LEIP in the breeding of many food microorganisms. The recent development of ion implantation as a novel way to introduce DNA element into the cells displays great potential in microbial breeding in the future (Gu et al. 2008).

Other techniques

The strain improvement for food microbes in the future is also possible with the use of other physical techniques to induce mutagenesis. For instance, a high-energy pulse electron beam (HEPEB)-mediated technology can trigger substantial DNA double-strand breaks (DSBs) with little effect on the cellular membrane integrity and enzymatic activity. Recent works on HEPEB show great potential in food microbiology, particularly in microbial breeding. For instance, this technique enhances the tolerance and ethanol production of *S. cerevisiae* (Zhang et al. 2012a, b, 2013). Another promising tool in microbial breeding is the application of laser technology. The technology generates heat, electricity, pressure, and magnet that allow energy accumulation of DNA molecules to an active state and subsequently trigger chemical and/or physical changes in the DNA, such as strand breaking or cross-linking (Liu et al. 2013; Yu et al. 2010).

Screening technology

Mutagenesis is just the first step in the microbial breeding process. Advanced mutagenesis technology creates innumerous mutants. However, an obvious gap between efficient mutagenesis and microbial strain screening makes identification of the mutants of interest from a mutated library much more challenging. Conventional screening relies heavily on manual isolation/purification and flask culture, which are laborious, tedious, and inefficient. While the microbial selection is relatively straightforward when choosing the obvious phenotype (e.g., antibiotic/nutrition/tolerance selective pressures), the selection of a nonobvious phenotype becomes more arduous, thus requiring new and efficient screening techniques. In the succeeding texts, we discuss current microbial screening technologies that show great potential in improving the selection of desirable food microorganisms.

Microtiter plate-based screening

To date, microtiter plate (MTP)-based screening technology is favored because of its moderate to high throughput and automation abilities and cost efficiency, making it a suitable

Table 3 Recent applications of LET-HIB for strain development in the food industry

Mutant(s)	Improvement	Reference
Aspergillus niger H4002	177.7 to 196.0 g/L citric acid was accumulated with the productivity of 3.0–3.3 g/L/h	Hu et al. (2014)
Aspergillus niger HW2	118.9 g/L citric acid was accumulated with the productivity of 2.2 g/L/h using cornstarch as feedstock	Hu et al. (2016, 2017a)
Aspergillus niger H11201	β-Glucosidase activity is 1340.4 U/mL, increased by 62.23%	Jiang et al. (2016a)
*Lactobacillusthermophilus*SRZ50	Productions of L(+)-lactic acid were increased to 23.2 g/L and 23.2 g/L using glucose and fructose as sole carbon sources	Hu et al. (2018)
Lactobacillus thermophilus A69	L(+)-lactic acid was accumulated to 114.2 g/L and 1.2 g/L/h, 16.2% higher than the starting strain	Jiang et al. (2018)
Aurantiochytrium sp. T-99	DHA was increased by 50% to 0.27 g/L/h and 30% to 27 g/L	Cheng et al. (2016)
Rhodotorula glutinis M5 and M16	Lipid contents were 28.8 and 30.7%, and lipid concentrations were increased by 76.5 and 91% to 0.60 and 0.65 g/L, respectively	Wang et al. (2009a)
Tricholoma matsutake Ar 59	A different colony morphology from the parent strain; stronger amylose- and cellulose-degrading activities to degrade water-insoluble amylase and cellulose substrates	Murata et al. (2018)

technology platform for preliminary screening (Long et al. 2014). A screening indicator is a key factor to allow the high sensitivity of MTP-based screening. General screening techniques usually use a single indicator based on the colorimetric assay (Rühmann et al. 2015). However, it is difficult to meet the demands for both high sensitivity and accuracy. A prevalent screening procedure is usually comprised of preliminary and secondary steps. The pH is a reliable and versatile indicator for the preliminary screening of those microbes that alter the pH or generate organic acids. Zeng et al. (2015) developed an HTS method based on pH change to select *Y. lipolytica* mutants with a high yield of α-ketoglutaric acid (α-KG). In the study, ARTP mutant strains undergo two rounds of separate screening using bromocresol and quinaldine red in MTPs. Another team develops a three-step HTS method that combines colony isolation, pH-sensing assay at A_{616}, and L-lactate oxidase (LOD) assay at A_{500}. They used a U-shaped deep-well MTP to screen the *B. coagulans* mutants overproducing lactic acid (Lv et al. 2016). Consequently, the technique allows the selection of 35 mutants with the desired phenotype from 750 mutant colonies and finally selects two mutants with the highest L-lactic acid yield. In addition, a 24-well U-bottom MTP with bromocresol purple as pH indicator enables the selection of L-lactic acid overproducers from a HIB mutant pool of *L. thermophilus* (Jiang et al. 2018). On the other hand, Shi et al. (2015) showed an efficient selection of *A. niger* mutant that overproduces gluconate using a 48-deep-well MTP. The researchers firstly screened mutants using a bromocresol green (a pH indicator), followed by a second screening using gluconate-chelated $CuSO_4$. This method selects three gluconate overproducers out of 1000 mutants. Based on the immobilization of pH-sensing carboxy fluorescein and the pH-insensitive reference sulfohodamin, an HTS method in MTP enables screening of microbes from milk and yogurt where in situ screening is usually difficult (John et al. 2003a). Moreover, pH indicators are used for the preliminary screening of mutants with enhanced enzyme production (Zhu et al. 2017) and reduced by-products (Zhang et al. 2017). The secondary screening commonly uses a colorimetric assay in selecting the specific characteristics of the product of interest.

Fluorescence-activated cell sorting

Flow cytometry (FC) enables counting, monitoring, enriching, and sorting of microbial cells suspended in fluid

Table 4 Recent applications of LEIP for strain improvement in the food industry

Mutant(s)	Improvement	Reference
Aspergillus niger N212	Xylanase was increased by 90.6% to 610 IU/mL; optimized fermentation temperature was increased to 30 °C	Li et al. (2007)
Aspergillus oryzae 100-8	Acid protease activity of 2834.6 U/g and neutral protease activity of 2601.0 U/g were achieved; activity of acid protease was enhanced about 44.1% at 36 h during koji fermentation; mutated mycelium was stronger and thicker	Zhao et al. (2012)
Bacillus amyloliquefaciens RL-1	Secreted and thermostable α-amylase activity was increased by 57.1% to 58.5 U/mL	Li et al. (2011)
Bacillus sp. S65F5	Chitosanase was increased by 6.1-folds to 25 U/mL; fermentation time was shorted to 56 h	Su et al. (2006)
Rhizopus arrhizus N1023	Lipase was enhanced by 165% to 175 μ/mL in a shaking flask culture	Ji et al. (2008)
Lactobacillus casei N-2	L(+)-lactic was improved by 38.8% to 136 g/L	Li et al. (2012b)
Schizochytrium sp. S1	DHA titer was boosted to 6.5 g/L, a 61% increase than the parent strain; DHA content accounted for 46.2% of total lipid	Fu et al. (2016)
Gluconobacter oxydans I-2-239	1,3-Dihydroxyacetone productivity was increased to 103.5 g/L, 115.7% higher than the wild strain; cultivation time was shortened to 36 h	Lin et al. (2016)
Wickerhamiella domercqiae var. *sophorolipid* N3-18 and others	104.5 and 135 g/L sophorolipids (SLs) were produced in a shake flask and a 5-L bioreactor, respectively, increased 84.7% than the parent; acidic SLs were increased 2.0-folds; diacetylated lactonic SL with a C18 monounsaturated fatty acid was boosted by 105.4%	Li et al. (2012a)
Phaffia rhodozyma E5042	Astaxanthin yield achieved to 2510 μg/g DCW in 50-L bioreactor, an increase of 125.5%	Liu et al. (2008)
Hansenula polymorpha HP28	GSH titer was increased by 1.6 times to 337.2 mg/L in shaking flasks Biomass was increased by 9.1% Fermentation time (in 500-mL flask) reduced to 42 h	Qian et al. (2013)
Rhizopus oryzae RQ4015	The titer and productivity of L(+)-lactic acid were increased by 10 and 46.7%, reached 121 g/L and ~3.4 g/L/h, respectively, using glucose as sole carbon source; over 80 g/L L(+)-lactic acid was produced using the mix of glucose (75 g/L) and xylose (25 g/L) produced	Wang et al. (2009b)

streams. FC technology uses fluorescence and microfluidic techniques for high-throughput characterization, identification, and screening of food microorganisms. Among them, fluorescence coupled with FC allows fast and real-time screening of the desired microbial strains. Fluorescent signals that correlate with cellular chemical or physical characteristics of the target strain enable microbial selection through fluorescence-activated cell sorting (FACS). For instance, FACS is applied in the screening of strains with a phenotype that overproduced the essential antioxidant glutathione (γ-glutamyl-L-cysteinylglycine, GSH). Selecting a mutant with a high content of GSH is critical. To date, yeast is the main GSH producer in the food industry. *S. cerevisiae* mutant G-143, a product of ethyl methanesulfonate (EMS) mutagenesis that produces high GSH content, is isolated based on the fluorescence intensity formed by intracellular GSH using monochlorobimane (mBCl) (Wang et al. 2010).

Flow cytometry-based technique using two nucleic acid stains, i.e., SYBR Green II RNA gel and propidium iodide, enables an accurate assessment and cell sorting of *Lactobacillus sakei* under heat and acid stress conditions (Bonomo et al. 2013). Through this protocol, fluorescent intensities and types could identify cells with different viabilities and conditions. In addition, a combination of FACS and fluorescence in situ hybridization (FISH) using a specific 16S rRNA probe labeled with fluorescein isothiocyanate (FITC) allows the identification and selection of functional acetic acid bacteria in vinegar. This procedure enables relatively short screen time to select strains that have high resistance to acetic acid and/or with a high yield of acetic acid from *Komagataeibacter*, *Acetobacter*, and *Gluconobacter* genera (Trček et al. 2016).

Aside from the heterogeneous probes, native molecular elements are also applicable for FACS. Vitamin B_{12} (VB_{12}) is widely used in the food industry with increasing demand in the global market. To date, mutagenesis is still a major means to enhance its production, so the selection for high-yield mutants becomes critical. Cai et al. (2018) developed an HTS system for isolating *Sinorhizobium meliloti* mutants with high VB_{12} content. In this system, the riboswitch RNA element of *btuB*, a key gene for VB_{12} biosynthesis, regulates the expression of GFP and *lacI* reporter system. This allows the identification and selection of cells based on the positive correlation between intracellular VB_{12} level and fluorescence intensity. Using this system, the same team isolates an ARTP mutant MC5-2 that is capable of producing VB_{12} by 21.9% higher than the wild-type strain.

Fluorescence-activated droplet sorting

Droplet microfluidics emerges as a screening technology with high-throughput and high-resolution characteristics. Picoliter-sized aqueous droplets are dispersed in a continuous fluorinated oil phase, allowing stable compartmentalization by surfactants. Theoretically, every variation can be detected and sorted based on its size and associated fluorescence.

Screening microbes producing enzymes represents a challenge, due to the lack of obvious phenotype and low efficiency of the conventional screening. Furthermore, current physiological and biochemical methods used in enzymatic assays are often laborious and expensive. Droplet microfluidics-based screening provides a solution as this tool enables fast screening rate (i.e., thousands of droplets per second) just by using a tiny reaction volume. Sjostrom et al. (2014) developed a fluorescence-activated droplet sorting (FADS)-based HTS to select yeast cells with improved secretion of α-amylase from a whole-genome mutated cell library. The method enables a saturated screening of the mutant library with a great reduction of reagent consumption. In a following study, researchers from the same group selected dozens of *S. cerevisiae* mutants with enhanced α-amylase secretion ability from UV mutagenesis libraries using two rounds of HTS based on FADS (Huang et al. 2015), demonstrating the reliability of their method. Whole-genome sequencing then revealed 330 mutation sites within 146 protein-encoding genes in the genome that are related to secretion modulation. Finding these unknown but critical loci and genes would have been impossible without the use of FADS.

For filamentous fungi such as *A. niger*, MTP-based screening is usually neither efficient nor cost-effective. Even FACS-based screening is also problematic because of the oversized *A. niger* mycelia. By contrast, FADS demonstrates promising results as an efficient technology for the HTS of filamentous fungi. Using the same fluorescein as the two studies mentioned above, a UV-mutated *A. niger* library with enhanced α-amylase activity is enriched by approximately 200-folds through FADS. In comparison with a UV-mutated reference strain, 37% of the sorted mutants show higher amylase activity (Beneyton et al. 2016).

In addition, FADS-based HTS also demonstrates a powerful capacity to screen lipase overproducers (Qiao et al. 2017), *p*-coumaric acid-overproducing *S. cerevisiae* strain (Siedler et al. 2017), or vitamin overproducer lactic acid bacteria (LAB) strain (Chen et al. 2017). A comprehensive review of its application for strain improvement of LAB has been published elsewhere (Chen et al. 2018). Although these examples were not specifically associated with mutagenesis, they demonstrate that droplet microfluidics-based screening is promising for strain improvement in the food industry.

Microscaled cultivation

Although mutant enrichment can be performed through HTS, the selection of the desired mutant microbe for fermentation remains a challenge. On one hand, a lack of kinetics

information in flask culture system would likely result in poor reproducibility. On the other hand, it is impossible to assess every mutant of interest in scaled bioreactors. Thus, microscaled cultivation is currently developed and applied in strain improvement for bioprocess monitoring and for control and optimization. Considering fermentation aims to producing at an industrial scale, desirable microscale fermentation needs to meet several basic criteria, such as scalability, reliability, and real-time monitoring. The principles and applications of microscale cultivation in pharmaceutical or industrial microorganisms have been reviewed (Long et al. 2014; Schäpper et al. 2009); hence, this review focuses only on their potential application and development in food microbes.

Online process monitoring

Process monitoring is crucial for fermentation. However, conventional monitoring is based on "off-line" techniques which require sampling out of a reactor to do measurement. In addition, pH and DO values are conventionally measured using electrodes that need calibration. However, modern fermentation processing concerns more about online and noninvasive monitoring with medium to high throughput than ever before. Since MTP-based cultivation can meet the demands of throughput, it is prevalent to develop novel monitoring techniques.

Mutant microbes are desirable materials to investigate intracellular metabolic flux. However, the flux tends to be susceptible by microbial growth status. The combination of microscale cultivation with molecular techniques allows the task to be easier. In lysine-producing mutants of *Corynebacterium glutamicum*, the MTP-based online monitoring associated with cultivation feeding ^{13}C isotope reveals that changes on kinetics significantly disturbed the metabolic flux for lysine production (Wittmann et al. 2004). Oxygen transfer rate (OTR) is a critical parameter for aerobic fermentation, but it greatly differs between regular MTPs and shaking flasks, and thus, this parameter is a major bottleneck for the scale-down or scale-up of microbial fermentation. A microscale system developed for the amino acid producer *Corynebacterium glutamicum* shows that biomass and specific growth rate parameters are comparable between MTPs and flask. The system involves a setup for both oxygen-sensitive and reference fluorophores that are immobilized at the bottom of wells and used a 96-well MTP to sense the concentration of dissolved oxygen (DO) (John et al. 2003b).

Recently, a MTP fermentation system called BioLector microbial bioreactor is available in the market. By means of scattered light and fluorescence optic measurement techniques, it can be used to analyze strain phenotype and growth, screen mutants, and/or optimize medium and other fermentation parameters. Compared with conventional bioprocessing tools, BioLector is labor and cost-effective and has high reproducibility. Infrared fluorescent oxygen-sensitive nanoparticle was demonstrated as a reliable method to monitor OTR of *Hansenula polymorpha* and *Gluconobacter oxydans* via BioLector-based respiration activity monitoring system (RAMOS) (Jang et al. 2017). The results prove that none of the fluorescence intensities, concentrations, and types disturbed the DO measurement by infrared fluorescence. However, RAMOS is only available in small-scale fermentation such as a flask or milliliter-scale microbioreactors. Flitsch et al. (2016) constructed a 48-well MTP-based μRAMOS device with reduced pneumatic valves and sensors but with a steady well-to-well integration. The cultivation of microbes such as *H. polymorpha* in this device shows a comparable OTR with the flask culture system. In contrast to a bioreactor, microscale cultivation shows higher efficiency with reduced cost and media consumption.

Process control and optimization

Microscaled cultivation is able to accelerate the optimization of biological and bioprocessing parameters with high throughput. By means of the BioLector microbioreactor platform equipped with the smart design of triggering sampling or dosing by pipetting robot, Rohe et al. (2012) quickly optimized fermentation conditions for high lipolytic activity in *C. glutamicum* after testing multiple parameters in a mean time such as pH, OTR, inoculation and induction time, specific growth rate, biomass, and enzymatic activity. *Aspergillus terreus* is a main producer for itaconic acid. Microscaled culture system performed using MTPs simplifies and speeds up the optimization process for itaconic acid production. The cultivation demonstrates the critical roles of $CuSO_4$ and KH_2PO_4, as well as the dispensability of nitrogen and phosphate for fermentation (Hevekerl et al. 2014).

Scalability and reliability are critical properties for microscale fermentation. In the examples mentioned above, Rohe et al. (2012) found that biomass accumulation and substrate utilization during microscale fermentation were quite similar to that when using a 1-L bioreactor. In 20-L bioreactors, both parameters reduced but still comparable to the smaller-scaled reactors, but the cutinase activities under the optimal conditions were comparable in all scales. Similarly, the results studied by Hevekerl et al. (2014) also demonstrated reliability and scalability of microscale fermentation since the bench-scale fermentation using the optimized medium showed high production of itaconic acid. However, MTP-based cultivation for some filamentous fungi remains problematic. The application of the Duetz-MTP system allows comparison of fungal fatty acid producers *Mucor circinelloides*, *Mo. alpina*, and microalga in various scales. Biomass accumulation, lipid content, and ARA production show relative scalability between MTPs, bench-top, and 25-L bioreactor, but the reproducibility varies between techniques (Kosa et al. 2018). In *A. niger*, 2-

phenylethanol (2-PE) production in FlowerPlate MTP cultivation shows a 35% reduction compared with that in flasks, even with the addition of homogenate chemicals (Huth et al. 2017).

Microfermentation

MTP-based system for aerobic fermentation relies on a special well (Funke et al. 2009), a lid (Schleputz and Buchs 2014), and external power to improve oxygen mass transfer. Alternatively, a milliliter-scale bioreactor is equipped with a novel stirrer that enables good mixing and high oxygen transfer rate (Bolic et al. 2016). Magnetic stirring and computational fluid dynamics (CFD) allow one- and bi-directional mixing and estimate the mixing time. This system generates high compatibility and flexibility (0.5–2 mL) in *S. cerevisiae* and *Lactobacillus paracasei*. In particular, this is also suitable in the case of filamentous fungi with high-viscosity fermentation. Further findings suggest that viscosity up to 35 mPa did not show any major influence on the oxygen transfer rate and the mixing process. The details on microscale cultivation technology and machines have been reviewed recently (Puskeiler et al. 2005).

In addition, MTP-based fermentation is developed for mixed cultured food. Ethanol and acetic acid levels produced by AAB are critical for the flavor of vinegar. The use of FlowerPlate BioLector allows a high-throughput system for bacterial selection and process investigation in a microscaled setup. Moreover, the design of a custom-made lid coupled to MTP cultivation permits simultaneous online monitoring, automatic sampling and measurement, efficient mass transfer, and prevention from evaporation. The MTP cultivation system could efficiently prevent evaporation of ethanol, acetic acid, and culture media. On top of that, the fermentation performance is comparable to that in a 9-L bioreactor (Schleputz and Buchs 2014).

Anaerobes are a special kind of food producers. So far, the study on miniaturized cultivation system for anaerobic fermentation is rare. A newly developed special MTP-based system, called OVAMO, uses Oxyrase, vacuum, and mineral oil to realize an in situ anaerobic environment in 96-well MTPs (Lam et al. 2018). This cost-effective system enables the growth of probiotics such as *Bifidobacterium longum* and carbohydrate monitoring in MTPs. The product obtained from OVAMO is comparable with that obtained from anaerobic jars.

Future perspectives

Although modern technologies for strain development are widely applied, some identified challenges could affect its potential application. These challenges may include the lack of specific mutagenesis devices and biosensing systems, the insufficient improvement of the mixed culture system, the low efficiency when using filamentous fungi and flocculating bacteria, and the insufficient safety assessment on harnessing genome-editing technology. Hence, strain improvement in the food industry still needs a lot of work in the future.

Combined mutagenesis

Repetitive treatments by a single mutagenesis technology would likely reduce mutation efficiency. For example, lactic acid production by *Sporolactobacillus* after the first round of ARTP treatment could boost by nearly 40%, while the second round could add around 11% on its production (Sun et al. 2015). Combined treatment is also efficient to improve the strain of food microbes. *Gluconobacter oxydans* is an outstanding example where the combined treatment with three mutagenesis techniques generates a significant overproduction of 1,3-dihydroxyacetone. The first process uses UV mutagenesis to select the overproducer strain U-2-115. This strain shows a 48.8% increase of 1,3-dihydroxyacetone production compared to the original one. The second process subjects the U-2-115 mutant into ARTP treatment generating a second mutant A-2-64, which can produce 26.3% more than the first mutant strain. The final process uses LEIP treatment to create the mutant strain I-2-239. This mutant strain shows dihydroxyacetone production of 103.5 mg/mL, i.e., 14.7 and 115.7% increase compared to the strain A-2-64 and the original strain L-6, respectively (Lin et al. 2016).

The combination of mutagenesis technology with other biotechniques shows some promising results as it works synergistically and efficiently. Jin et al. (2018) created an engineered *S. cerevisiae* strain via ARTP mutagenesis and metabolic engineering. This approach results in high astaxanthin yield of up to 10.1 mg/g dry cell weight (DCW), which is so far the highest yield recorded from a yeast culture using a flask. In *B. coagulans*, mutants derived from ARTP and ALE treatment can grow in a medium with 80% diluted acid hydrolysates (Jiang et al. 2016b). Moreover, genome shuffling coupled with mutagenesis is also reported to improve the breeding of food microbes (Zhang et al. 2015a).

Development of biosensing systems

Undoubtedly, the biosensing system plays a central role for screening and microscale cultivation for being high throughput, highly sensitive, and reliable and for its ability to monitor microbial culture in real time. The type, development, and potential use of biosensors are reviewed elsewhere (Gredell et al. 2012; Han et al. 2018; Johnson et al. 2017; Mertens and Liese 2004; Schallmey et al. 2014; Shibasaki and Ueda 2014; Zhang et al. 2015b). So far, there are very few progresses on the use of biosensors in the food industry for sensing and/or selecting pH (Casimero et al. 2018), oxygen and cellular viability(Strianese et al. 2009), aroma (Liu et al. 2015), or

amino acid producers (Liu et al. 2017a; Mustafi et al. 2012), lagging behind the actual needs of the food industry. So far, real applications of biosensing systems in the industry are rare, but they would be promising in the near future, in particular in pharmaceutical or biotechnological plants producing high value-added products, which have been addressed in recent reviews (Neelam et al. 2019; Lam et al. 2012). However, they have not been widely applicable in the food industry yet. We can predict that microscale fermentation-equipped biosensing systems will be available for screening and process optimization in the food industry, but their cost will be critical to apply in the food industry.

Strain improvement for special food microorganisms

Traditional fermented food by mixed cultures

Mixed microbial cultures play a substantial role in the production of traditional food, including Chinese liquor, pickles, soy sauce, and fermented soya. Because of the great economic, nutritional, and cultural values offered by traditional food, the improvements in their nutritional contents and flavors are increasingly attractive. However, efficient mutagenesis and screening techniques for mixed cultures are much more difficult than for pure microbial cultures, because the mixed cultures form a consortium in which cellular interaction, evolution, and survival are different from pure cultures, and such a complicated consortium rather than a certain individual member confers food diverse flavors, metabolites, and so on. The functions of the microorganisms in mixed cultures are different, so it is hard to unify the mutation and screening directions. Meanwhile, different microorganisms in mixed cultures have different sensitivities to mutation factors, and it is difficult to screen a suitable mutation technique for mixed microbe mutation. A compromised mutagenesis strategy for mixed culture is to mutate a few cultured members and then acclimatize them to a mixed culture condition (Zhang et al. 2015a). However, the strategy does not consider the mixed cultures as an organic whole and neglects the contribution of uncultured or nonisolated microbes in the mixed cultures. In addition, it is infeasible to uncultured but functional species. A desirable mutagenesis technology for mixed cultures should enable in situ and efficient mutation of all microbial members at one time. Therefore, improvement of the mutagenesis techniques must involve the following modifications: treating a larger cell amount, optimizing the treatment time and strength, and maintaining steady mutation efficiency in a solution. On the other hand, HTS will be a primary requirement for mixed cultures though it will be challenging to perform. A recent report on the use of droplet-based microfluidics for the HTS of novel enzyme resources from metagenomic library provides a promising strategy on screening mixed cultures of food microbes (Hosokawa et al. 2015).

Screening and microscale cultivation of filamentous fungi and flocculating bacteria

Filamentous fungi and flocculating bacteria are especially important for fermented food because of their high yield, high tolerance, and easy recovery. However, the formation of biomass agglomerates or flocs makes the screening and assessment more challenging. Oversized pellets and/or uneven phase in the solution constrain the application of screening methods, which are dependent on optical density, surface characteristics (e.g., using FACS), and droplet microfluidics-based technology. Since filamentous fungi are vulnerable to mechanic shearing and tend to generate high viscous culture broth during fermentation, the scalability of microcultivation for fungi still requires improvement. To partly address this concern, a recent study creates a three-dimensional model to stimulate the hyphal growth and OTR of the filamentous fungus *Rhizopus oligosporus* at a microscale and solid-state fermentation (Coradin et al. 2011). Hence, microscale cultivation significantly guides in understanding any specific fungi even though its robustness remains to be tested.

The genome editing and breeding of food microorganisms

Genome-editing technology using the CRISPR system is a recent powerful tool (Doudna and Charpentier 2014; Hsu et al. 2014). Since it introduces no foreign genetic elements into the host genome, it is regarded as safe and different from genetic modification (GM). So far, most applications of the CRISPR technique were reported in LAB or yeast. Using such technique could efficiently enhance the production of food-grade lactic acid (Jang et al. 2017) or *N*-acetylglucosamine (Zhou et al. 2019) without introducing antibiotic markers or heterogeneous DNA. During yeast breeding, CRISPR enabled to enhance thermotolerance (Mitsui et al. 2019) or reduce its foam of sake (Ohnuki et al. 2019). In addition, researchers also demonstrated the efficacy and feasibility of applying CRISPR in microbial populations. Barrangou and Notebaart (2019) and Goh and Barrangou (2019) have reviewed advances on using CRISPR techniques to engineer probiotics for the aim of therapy and health, or reshaping microbial populations of the food supply for food safety. In addition, there were reports on developing CRISPR tools in edible mushroom (Binhu and Das 2019) or *Lactobacillus plantarum* and *Lactobacillus brevi* (Huang et al. 2019), displaying great potential for strain development in the food industry. However, the study of Leenay et al. (2019) indicated that the precision of CRISPR varied and was likely host-dependent. This result reminds us again to be cautious to the food safety and risk management by the genome-editing techniques (Mays and Nair 2018; Varela and Varela 2019).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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