# Public version of the Final Report of TKI project (TKIBE01001) Novel Strain Improvement Strategies for Industrial Microorganisms (ISIM)

*This final report includes a summary of highlights of the results, conclusions and recommendations of the ISIM project, coordinated by BE-Basic and funded by the Dutch TKI initiative*

## 1. Characteristics of the ISIM project

| | |
|---|---|
| Project number: | TKIBE01001 |
| Project title: | Novel Strain Improvement Strategies for Industrial Microorganisms |
| Project acronym: | ISIM |
| Project Coordinator: | BE Basic Foundation |
| Project partners: | University Groningen |
| | TU Delft |
| | Wageningen UR |
| | NIZO Food Research |
| | Friesland Campina NV |
| | CSK |
| | Chr. Hansen |
| | Heineken NV |
| Start date project: | 15-10-2012 |
| End date project: | 31-03-2019 |
| Date final report: | 31-08-2019 |
| Subsidy: | € 2.015.044 |
| Own contribution: | € 590.817 |
| Total Program | € 2.605.861 |

*More copies of this public version of the ISIM report are freely available from:*

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# 2. Final Report on Results Achieved

## 2.1. Problem definition in relation to aims of the subsidy program: program line: biotechnological conversion technologies

This project generates knowledge and develops appropriate non-GMO engineering tools to introduce desirable traits (e.g., increased product tolerance to alcohols and biofuel liquids) into industrial host organisms for applications in fields of food & crop improvement, bioremediation and bioconversion of feedstocks for production of biobased chemicals and fuels. In view of the persisting public opinion and attitude towards the use of GMOs in e.g., foods and environment, and the current legislative environment, there is an imminent need to develop methods that allow the introduction of DNA encoding any desirable trait through a non-GMO method into food-grade and other industrial host organisms of choice. Although this industrial need has been recognized for decades, to date no concerted actions have been undertaken to develop the appropriate non-GMO engineering tools in a structured and result-oriented way. The GMO issues do mostly affect the developments in the European food-market, but do also extend to other areas in agriculture and biobased economy, where bacteria or other microorganisms are used in fermenters, or open field applications for some areas of major economic and societal importance. In order to achieve this goal, four major approaches have been selected as the core technologies to develop the non-GMO engineering deliverables, namely conjugation, genetic competence, phage transduction (for lactobacillae (LAB), random mutagenesis and laboratory evolution (in bakers' yeast). These approaches are encompassed within 5 interactive PhD projects.

The 5 PhD projects were organized in two BE Basic projects:

**F10.002.01 –** Non-GMO methodologies for introducing desirable traits in food-grade LAB and Bacilli (ISIM I), using conjugation, genetic competence and phage transduction within PhD-projects 1-4:

1. Natural gene transfer to LAB by employing natural competence (Joyce Mulder)

2. Develop new and natural tools to introduce desirable traits into *Lactococcus lactis:* a starter strain (Barbara Marcelli)

3. Natural methodologies for introducing desirable traits in food-grade LAB and *Bacilli. L. lactis* ICE presence and variations (Simon van der Els)

4. *Bacillus subtilis* as an intermediate host for transfer of DNA to other Bacilli or to Lactococci (Luiza Morawska)

**F10.002.02** - Novel evolutionary engineering strategies for yeast (ISIM 2) using random mutagenesis, combined with existing and novel approaches for laboratory evolution in Yeast (PhD-project 5)

5. Novel evolutionary strategies to improve product tolerance in yeast (Arthur Gorter de Vries)

## 2.2. Highlights of Research & Development

*Here we present a summary of selected highlights obtained in each sub-project. For a full overview of results we refer to the annual reports 2014-2019.*

**PhD-project 1 Natural gene transfer to LAB by employing natural competence;**

Horizontal gene transfer (HGT) plays an important role in the natural evolution of bacteria. An important mechanism in HGT is natural competence, a state in which bacteria can internalize exogenous DNA, followed by autonomous replication either as plasmid, or introduced in the chromosome. In this PhD project a successful approach for introduction novel traits into lactic acid bacteria (LAB) was achieved through induction of natural competence in complete strains of L. Lactis. This was achieved by overexpression of the master regulator of competence ComX. Importantly, work has focusses on elucidation of the natural, environmental trigger involved in competence activation in *L. lactis*, using *com*-promoter reporter protein fusions to detect inducing conditions using high throughput screening models. These high throughput experiments are complemented by molecular tracking in single lactococcal cells to detect and visualize low-frequency competence activation events. This work has contributed importantly to a review published by Bron et al (2019) on DNA transfer strategies for improvement of industrial lactic acid bacteria and a PhD dissertation is in preparation.

**PhD-project 2 Develop new and natural tools to introduce desirable traits into *Lactococcus lactis* a starter strain;**

The main goal of this project is to exploit phage transduction as a tool for genome editing in different strains of *L .lactis* and other Lactic Acid Bacteria. Over 30 new phages and their hosts were identified and characterized by sequencing and genomic analyses. Moreover, a rare phage was identified that is now further characterized on transduction properties. In the year 2017, thirty-two phage genomes were thoroughly analyzed with the main aim of identifying the best phage candidates to use in the transduction experiments. The genomes sequences provided information about the replication type of the phages (*pac* or *cos*) and about the possible presence of integrated bacterial DNA. Phages with a *pac* type replication and/or with traces of bacterial DNA in their genomes were considered to be more prone to perform generalized or specialized transduction and so were the main target of the research. Host range and phage-type determination by analysis of C2 phages revealed that no precise correlation exists between phage type and host range. The establishment of a phage-mediated horizontal gene transfer protocol in *L. lactis* was achieved and plasmid DNA transfer succeeded with 3 phages and 2 different plasmids. A PhD dissertation is in preparation.

**PhD-project 3 Natural methodologies for introducing desirable traits in food-grade LAB and *Bacilli. L. lactis* ICE presence and variations;**

In this project the main aim was to study the use of Integrative conjugative elements (ICE), which are autonomous mobile genetic elements encoding their own mobilization and transfer machinery, to facilitate horizontal gene transfer from donor to recipient strains. Besides the canonical mobilization and transfer machinery, ICE often encode accessory genes that provide their host with an advantage under certain environmental conditions. These accessory genes encode a variety of functions, including several that are of potential industrial relevance like specific carbohydrate utilization, phage resistance, and bacteriocin production. Using an *in silico* search engine 25 ICE were identified in *L. lactis* genomes. A Cas9 mediated curing protocol is presently exploited to proof ICE functionality and simultaneously eradicate them from the host genome to define the precise ICE boundaries to pinpoint the accessory genes they encode. In parallel, comparative genetic analysis of the ICE identified revealed at least three ICE families in *L. lactis* for which we are currently

determining their donor-recipient host-range, using selectable accessory gene functions to select transconjugants. From the model ICE Tn6098 we selected 24 candidate genes for constitutive expression. This set included genes of both known and unknown functions. The effect of constitutive expression on ICE excision and transfer rates was measured. None of the constructs showed a major effect, and there is no viable correlation between excision and transfer efficiency established. Furthermore, we created a construct expressing the *excisionase* gene (which is expected to play a major role in ICE excision) under the nisin promoter. Upon induction using nisin, we observe an immediate halt in cell growth. Further analysis of these cells implicate that excisionase has pleotropic effects when highly expressed.
We validated a range of fluorphores (both constitutive and photoactivatable) in *L. lactis* and demonstrated their applicability for future microscopic research. Furthermore, we optimized and developed methods to perform single-molecule tracking photoactivated localization microscopy in L. lactis (stPALM).These methods were then further optimized and used for a study of dCas9 tracking and quantification in L. lactis. A PhD dissertation is in preparation

**Sub-project 4 *Bacillus subtilis* as an intermediate host for transfer of DNA to other Bacilli or to Lactococci;**

In this project *Bacillus subtilis* is employed as an intermediate host to facilitate transfer of DNA and proteins to other bacteria. Proof of concept for DNA transfer between *Bacillus subtilis* and LAB was achieved using an optimized mating protocol that enabled the exchange of small sized plasmids. Using this method we found that *B. subtilis* and *L. lactis* can efficiently transfer plasmid DNA, at a frequency of up to $10^{-4}$. Moreover, we also observed the transfer of small proteins (like GFP) via non-conjugative cell-to-cell contact.
In this project we were also focused on natural genomic DNA transfer and for this purpose a combinatory method for genomic DNA fragmentation and its transfer via nanotubes was designed.
Nanotubes are able to transfer proteins between species. Using this knowledge we designed protocol for competence induction in non-competent *Bacillus subtilis* 168 (Δ*comK*) using donor strain of *Bacillus subtilis* and *Lactococcus lactis* with inducible comK. Using fluorescent microscopy we could observe the competence induction in Δ*comK* strain. A PhD dissertation is in preparation.

**Sub-project 5 Novel evolutionary strategies to improve product tolerance in yeast;**

There is increasing interest in the use of yeast to produce alcohols ranging from C2 to C5 such as ethanol (C2), and iso-butanol (C4) for use as biofuels. The production of iso-butanol is under particularly intensive study, because its physical and chemical properties offer significant advantages over those of ethanol. A major obstacle for industrial production is the low tolerance of S. cerevisiae to even small concentrations of the product: levels of 1-2% of iso-butanol are already toxic. Thus, improving tolerance to alcohols is essential to allow production of next generation biofuels at industrially relevant levels without having to resort to complex, costly fermentations setups. While the tolerance of S. cerevisiae to ethanol has been explored extensively over the past decade, its tolerance to higher alcohols remains largely unexplored. It is likely that there is not a single genetic determinant for this phenotype, but rather that a number of interacting factors - a combination of single nucleotide variations, structural variation of varying lengths and copy number variation - will play a role. In this project we will develop tools for high-throughput mining of natural genomic variation (found in strain collections) and induced genomic variation (found by e.g. mutagenesis or evolutionary engineering) to discover the key genetic determinants underlying tolerance to higher alcohols. The end goal is to reverse engineer these determinants to improve tolerance of S. cerevisiae to iso-butanol, allowing to achieve industrially relevant titers. While the project will focus on iso-butanol for reasons of relevance to current industrial needs, the bioinformatics and molecular tools developed in this study will be applicable to any desired phenotype, not solely restricted to product tolerance.

Nanopore sequencing and comparative genome analysis confirm lager-brewing yeasts originated from a single hybridization. Our findings suggest that Group 1 and Group 2 strains originated from a single hybridization involving a heterozygous *S. cerevisiae* strain, followed by different evolutionary trajectories. The clear differences between both groups may originate from a severe population bottleneck caused by the isolation of the first pure cultures. Alpaca provides a computationally inexpensive method to analyse evolutionary relationships while considering non-linear evolution such as horizontal gene transfer and sexual reproduction, providing a complementary viewpoint beyond traditional phylogenetic approaches.
Use of Alpaca, a kmer-based approach for investigating mosaic structures in microbial genomes.
Microbial genomes are often mosaic: different regions can possess different evolutionary origins due to genetic recombination. The recent feasibility to assemble microbial genomes completely and the availability of sequencing data for complete microbial populations, means that researchers can now investigate the potentially rich evolutionary history of a microbe at a much higher resolution. Here, we present Alpaca: a method to investigate mosaicism in microbial genomes based on kmer similarity of large sequencing datasets. Alpaca partitions a given assembly into various sub-regions and compares their similarity across a population of genomes. The result is a high-resolution map of an entire genome and the most similar scoring clades across the given population.
Maltotriose consumption by hybrid *Saccharomyces pastorianus* is heterotic and results from regulatory cross-talk between parental sub-genomes The hypothesis that the maltotriose-positive phenotype in *S. pastorianus* is a result of heterosis was experimentally tested by constructing a *S. cerevisiae* x *S. eubayanus* laboratory hybrid with a complement of maltose-metabolism genes that resembles that of current *S. pastorianus* strains. The ability of this hybrid to consume maltotriose in brewer's wort demonstrated regulatory cross talk between sub-genomes and thereby validated this hypothesis. These results provide experimental evidence of the evolutionary origin of an essential phenotype of lager-brewing strains and valuable knowledge for industrial exploitation of laboratory-made *S. pastorianus*-like hybrids.
These results represent a first genomic and physiological characterization of maltose transport in *S. eubayanus* CBS 12357T and provides a valuable resource for further industrial exploitation of this yeast. A PhD dissertation has been published and defended.

## 2.3 Conclusions and Recommendations

➢ Based on the results obtained in project 1, it is concluded that several of the developed methods show good promise for application in introducing desirable traits into bacterial strains via a non-GMO strategy for natural gene transfer. and thereby fulfilling essentially and successfully a key aim of this ISIM program:

- A successful strategy was developed to induce the competence state in L. lactis which opened new options to create mutants within 1-2 days instead of weeks;
- A phage-mediated horizontal gene transfer protocol was established in L. lactis, showing successful plasmid DNA transfer;
- A successful donor-recipient conjugative transfer of 2 known composite transposons to L lactis was established, using integrated conjugation elements (ICE)
- A method was developed to perform single-molecule tracking from donor to recipient using fluorophore-based photoactivated localization microscopy in L. lactis

- Successful non-conjugative interspecies plasmid transfer, and protein transfer via cell-to-cell contact through nanotubes by a mating protocol for B subtilis, L. lactis and S. thermophilus

- These developed horizontal gene transfer approaches in food-grade LAB and B. subtilis and S. thermophilus enable the development of Industrially relevant strains with desirable traits in a non-GMO manner, which is a key area of development and primary research for European industrial parties in food, dairy, environment and biobased economy areas.

- Based on results obtained in project 2, improved understanding of the genome complexity and plasticity was achieved in the yeast strain S. pastorianus, which will enable simplifying and accelerating non-GMO strain improvement strategies such as mutagenesis and laboratory evolution in a eukaryotic microorganism.

- An efficient GM-based CRISPR-Cas9 strategy for genome editing was developed in laboratory and industrial yeast strains

- However, this CRISPR-Cas9 based gene editing tool, also created an unwanted loss of heterozygosity upon editing of heterozygous loci in S. pastorianus.

- This observed phenomenon of loss of heterozygosity, although valued as beneficial for generation of a wider genetic and phenotypic diversity of e.g. lager brewing hybrids, may have implications for CRISPR-Cas9 based human gene editing and therapy because the loss of heterogenicity can lead to cancer

- Overall it is concluded that the work executed in project 2 has resulted in GMO and non-GMO methods which can successfully be applied directly to improve and diversify yeast strains for Industrial applications in e.g., lager brewing, but also for improving tolerance of Saccharomyces sp. to alcohols important for biobased biofuel production.

- It is recommended to be careful when applying tools such as CRISPR-Cas9 for gene editing in higher eukaryotes and humans, due to the potential adverse consequences of loss of heterozygosity

- It is recommended that the Industrial parties make their Industrial strains easier available to the university partners within a public-private partnership collaboration; in this program it appeared that the material transfer agreement (MTA) regulations hampered or prohibited e.g., the sequencing of promising industrial strains.

# 3: Execution of the Program

## 3.1 Challenges & Technical Problems

During the execution of the ISIM sub-projects several challenges and bottlenecks were encountered, some of which could not be solved and require further investigations, while in other cases bypasses were generated. We were confronted with the following major bottlenecks:

Sub-project 1 by Joyce Mulder not achieved

- Natural competence in *L. lactis:* Restoration of late competence genotype in incomplete *L. lactis* strains
- Natural competence in *S. thermophilus:* Improvement of *S. thermophilus* competence and genetic pre-enrichment prior to transfer

Sub-project 2 by Barbara Marcelli not achieved

- Because of MTA regulations, promising industrial strains could not be sequenced. 17 *L. lactis* strains form the MolGen collection were sequenced and used for the screenings
- No chromosomal DNA transfer was observed with the phages and experimental condition tested

Sub-project 3 Simon van der Els not achieved

- List of possible transferable genomic elements in *S. thermophilus* genomes based on *in silico* screening
- Phages of *S. thermophilus* were not investigated

Sub-project 4 by Luiza Morawska not achieved

- Establishment of >2 food-grade selectable markers and traits that can be transferred into naturally competent *B. subtilis* strains
- Genome sequences of at least 15 *Bacillus subtilis* strains have been analyzed by bioinformatics for rudiments of phages, conjugative elements and competence genes
- Transduction experiments to test the ability of selected phages to transfer exogenous genetic have been completed, from *S. thermophilus* to *L. lactis*

## 3.2 Knowledge dissemination, output

Dissemination of results of the ISIM program were mainly achieved by academic versions of dissemination, like publications in peer-reviewed Journals, presentations, abstracts and posters presentations on International congresses and symposia. In addition, several parts of the results achieved in subprojects 3 and 5 were directly communicated with the Industrial partners involved and have been adopted, integrated and/or patented. In total 17 publications in peer reviewed Journals were achieved; 5 PhD theses have been completed or are near completion. A list of publications is given below.

Table 2: Output: list of papers from the ISIM program

| | **ISIM program: Academic publications in peer-reviewed journals and books** | | | | |
|---|---|---|---|---|---|
| **project** | **Author(s)** | **Title** | **Journal** | **Vol.-pp.** | **year** |
| 1 | Marcelli B, De Jong A, Karsens H, Janzen T, Kok J, Kuipers OP | A specific sugar moiety in the Lactococcus lactis cell wall pellicle s required for infection by CHPC971, a member of the rare 1706 phage species. | Applied and Environmental Microbiology | Jul 26 Epub | 2019 |
| 1 | van Beljouw, S. P. B., van der Els, S., Martens, K. J. A., Kleerebezem, M., | Evaluating single-particle tracking by photo-activation localization microscopy (sptPALM) in | Physical Biology | 16, 3, 1 p | 2019 |

| | ISIM program: Academic publications in peer-reviewed journals and books | | | | |
|---|---|---|---|---|---|
| **project** | **Author(s)** | **Title** | **Journal** | **Vol.-pp.** | **year** |
| | Bron, P. A. & Hohlbein, J. | Lactococcus lactis | | | |
| 1 | Bron, P. A., Marcelli, B., Mulder, J., van der Els, S., Morawska, L. P., Kuipers, O. P., ... Kleerebezem, M. | Renaissance of traditional DNA transfer strategies for improvement of industrial lactic acid bacteria. | Current Opinion in Biotechnology | 56, 61-68. https://doi.org/(...)j.copbio.2018.09.004 | 2019 |
| 1 | Mulder, J., Wels, M., Kuipers, O. P., Kleerebezem, M., & Bron, P. A. | Induction of Natural Competence in Genetically-modified Lactococcus lactis. | Bio-Protocol | 8(13), [2922]. https://doi.org/10.21769/BioProtoc.2922 | 2018 |
| 1 | van der Els, S., James, J. K., Kleerebezem, M. & Bron, P. A | Versatile Cas9-driven subpopulation selection toolbox for Lactococcus lactis | Applied and Environmental Microbiology | 84, 8, e02752-17. | 2018 |
| 1 | Mulder, J., Wels, M., Kuipers, O. P., Kleerebezem, M., & Bron, P. A. | Unleashing Natural Competence in Lactococcus lactis by Induction of the Competence Regulator ComX. | Applied and environmental microbiology | 83(20), [e01320-17]. https://doi.org/10.1128/AEM.01320-17 | 2017 |
| 2 | Gorter de Vries AR, Koster CC, Weening SM, Luttik MAH, Kuijpers NGA, Geertman JMA, Pronk JT, Daran JMG | Phenotype-independent isolation of interspecies Saccharomyces hybrids by dual-dye fluorescent staining and fluorescence-activated cell sorting | Frontiers in Microbiology | Vol 10; art 871 doi: 10.3389/fmicb.2019.00871 | 2019 |
| 2 | Salazar AN, Gorter de Vries AR, van den Broek M, Brouwers N, de la Torre Cortes P, Kuijpers NGA, Daran JMG, Abeel T. | Nanopore sequencing and comparative genome analysis confirm lager-brewing yeasts originated from a single hybridization | bioRxiv | Doi.org/10.1101/603480 | 2019 |
| 2 | Brouwers N , Gorter de Vries AR, van den Broek M, Weening SM, Elink Schuurman TD, Kuijpers NGA, Pronk JT, Daran JM | *In vivo recombination of Saccharomyces eubayanus maltose-transporter genes yields a chimeric transporter that enables maltotriose fermentation* | PLoS Genet | *15(4) :e1007853* | 2019 |
| 2 | Gorter de Vries AR, Voskamp MA, van Aalst ACA, Kristensen AL, Jansen L, van den Broek M, Salazar AN, Brouwers N, Abeel T, Pronk JT,Daran JMG | *Laboratory Evolution of a Saccharomyces cerevisiae × S. eubayanus Hybrid Under Simulated Lager-Brewing Conditions* | Front Genet | *10:242* | 2019 |
| 2 | Brickwedde A, Brouwers | *Structural, physiological and* | Front Microbiol | *9:1786* | 2018 |

| | ISIM program: Academic publications in peer-reviewed journals and books | | | | |
|---|---|---|---|---|---|
| **project** | **Author(s)** | **Title** | **Journal** | **Vol.-pp.** | **year** |
| | N, van den Broek M, Gallego Murillo JS, Fraiture JL, Pronk JT, Daran JMG | *regulatory analysis of maltose transporter genes in Saccharomyces eubayanus CBS 12357T.* | | | |
| 2 | Salazar AN, Abeel T | *Approximate, simultaneous comparison of microbial genome architectures via syntenic anchoring of quiver representations* | Bioinformatics | *34:i732-i742* | 2018 |
| 2 | Gorter deVries AR, Couwenberg LGF, van den Broek M, de la Torre Cortes P, ter Horst J, Pronk JT, Daran JMG | Allele-specific genome editing using CRISPR-Cas9 is associated with loss of heterozygosity in diploid yeast | Nucleic Acid research | 1-11 doi: 10.1093/nar/gky1216 | 2018 |
| 2 | Juergens H, Varela J, Gorter de Vries AR, Perli T, Gast V, Gyurchev N, Rajkumar A, Mans R, Pronk JT, Morissey J, Daran JMG | Genome editing in Kluyveromyces and Ogataea yeasts using a broad-host-range Cas9/gRNA co-expression plasmid | FEMS Yeast Research | FEMSYR-17-10-0192.R1 | 2018 |
| 2 | Gorter de Vries AR, de Groot PA, van den Broek M, Daran JMG | CRISPR-Cas9 mediated gene deletions in lager yeast *Saccharomyces pastorianus* | Microbial Cell Factories | *16:222 doi.org/10.1186/s12934-017-0835-1* | 2017 |
| 2 | Salazar AN, Gorter de Vries AR, van den Broek M, Wijsman M, de la Torre Cortés P, Brickwedde A, Brouwers N, Daran JG, Abeel T. | *Nanopore sequencing enables near-complete de novo assembly of Saccharomyces cerevisiae reference strain CEN.PK113-7D* | FEMS Yeast Res | *17:fox074* | 2017 |
| 2 | Gonzalez-Ramos, D, Gorter de Vries AR, Grijseels SS, van Berkum MC, Swinnen S, van den Broek M, Nevoigt E, Daran JM, Pronk JT, van Maris AJA | A new laboratory evolution approach to select for constitutive acetic acid tolerance in *Saccharomyces cerevisiae* and identification of causal mutations | Biotechnol. for Biofuels | 9; 173, 18p | 2016 |