Project Information Reporting Format for Agriculture, Water, and Food Public-Private Partnerships

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From the project plan (fill in as much as possible)

1. Project Information

1.1 Funding	PPS toeslag TKI-A&F
1.2 Project number	AF18141
1.3 Project title	A view to a cell
1.4 Project leader *(Name and e-mail address)*	Prof. Dr. Ruud A. Weusthuis, chair group Bioprocess Engineering ruud.weusthuis@wur.nl
1.5 Start date (*dd-mm-yyyy)*	1-4-2019
1.6 End date (*dd-mm-yyyy)*	31-12-2020
1.7 MMIP	D2 Gezonde voeding een makkelijke keuze

2. Project Description

2.1 Summary *Provide a brief summary of what the project entails and aims to achieve. It is a publicly available summary (target, contribution to the mission, results to be delivered in terms of knowledge for target group x and the partners in the project).*

Breast milk is the best nutrition for new-born infants. In cases where breast milk is not or insufficiently available, FrieslandCampina Domo wants to offer the highest quality infant formula fulfilling the nutritional needs of the infant. Oligosaccharides are a vitally important component of human milk, that are hardly present in cow's milk. Therefore, infant formula based on cow's milk is currently lacking human milk oligosaccharides. FrieslandCampina Domo is dedicated to developing human milk oligosaccharides for application in infant formula. Currently, over 200 human milk oligosaccharides (HMOs) have been identified in human milk. 2'-Fucosyllactose (2'-FL) is the most abundant oligosaccharide in human milk, making up 20 to 30% of all oligosaccharides in human milk. With Aequival® 2'-FL, FrieslandCampina Domo offers the first human milk oligosaccharide (not derived from human milk) for application in next generation infant formula. 2'-FL is produced by fermentation, using *E. coli* as microbial cell factory.

The goal of this project was to gain more insight in the mechanisms of the *E. coli* fermentation process yielding 2'-FL and to identify and validate potential improvement routes that lead to higher 2'-FL yields. Several work packages and tasks were performed with the following objectives

- Identify the metabolic bottlenecks that prevent more substrate going into the 2'-FL production pathway.
- Improving productivity and yield of 2'-FL
- Determine the stability of expression of the 2'-FL pathway genes and – if necessary – improve it.

- Identify how acetate is produced and suggest genetic interventions to reduce acetate production

2.2 Project target *What will the project contribute to the objectives of the KIA, the missions, and the MMIPs?*

The efficient production of 2'-FL contributes to the aim of MMIP D2 to produce a healthy and sustainable food supply

2.3 Motivation *Describe why this project is appropriate and necessary within the MMIP.*

This project entails making a product (Aequival®) and its optimized production process available to supply a healthy food component.

2.4 Result *Describe the intended results of the project as SMART as possible. These include results in terms of content (regarding question 2.2) and results such as meetings and reports. Include the timeline per year whenever possible.*

<u>WP1. Metabolic model</u>

Task 1.1. Conceptual metabolic model

A conceptual model linking substrate consumption to product formation by redox cofactors and energy generation.

Task 1.2. Genome-scale metabolic model (GEM)

A genome-scale metabolic model of *E. coli* with the reactions necessary for 2'-FL production.

Task 1.3. Identification of bottlenecks

Bottlenecks preventing higher 2'-FL production in the current production strain

Task 1.4. Model verification

A verified model using existing and new fermentation data from WP2 to WP4.

Task 1.5. Genetic modifications

The selected genetic modifications from task 1.3., realized by Glycosyn.

Task 1.6. Strain evaluation

The genetically modified strain evaluated by FrieslandCampina.

<u>WP2. Physiological studies</u>

Task 2.1. Medium optimization

A medium composition resulting in improved 2'-FL-production.

Task 2.2. Transcriptome analysis

Genes identified to be involved in the production of 2'-FL

Task 2.3. Limited metabolomics

Knowledge about availability of selected intermediates of 2'FL-production

Task 2.4. Genetic modifications

Proposed genetic modifications, to be realized by Glycosyn.

Task 2.5. Alternative cultivation protocols

Alternative cultivation protocols to improve 2'-FL production. To be assessed by Friesland Campina.

<u>WP3. Stability of expression</u>

Task 3.1. Determining the 2'-FL production capacity during fermentation process

The capacity of the production strain to produce 2'-FL during a fed-batch fermentation.

Task 3.2 and 3.3. Stability of expression of the 2'-FL pathway

Stability of expression of the genes of the 2'-FL pathway during the fermentation

Task 3.4. Options for genetic modifications

Options for genetic modifications to improve expression of the genes involved in the 2'-FL pathway.

Task 3.5. Strain evaluation

Effect of genetic modifications (realized by Glycosyn) on 2'-FL production is evaluated (by FrieslandCampina).

WP4. Acetate formation

Knowledge about acetate production by *E. coli* and a proposal for genetic engineering of the 2'-FL production strain to reduce acetate production.

Annual Report (please also fill this in for the final year)

3. Project Status

3.1 Project status *(select one option)*	Project is completed
3.2 Explanation including predicted changes to the original work plan	Project is completed. Metabolomics and transcriptomics were in first instance moved from WP2 to WP3 in agreement with all partners involved in the project. However, ultimately these analyses were not performed due to the COVID-19 pandemic. The allocated material budget was not used. The tasks 3.1, 3.2 and 3.3 were combined.

4. Achieved results

4. Brief description of the results and their contribution to the MMIP (as described in 2.2)

WP1. Metabolic model

Task 1.1 – Conceptual model

A conceptual model was realized in which all substrates and products involved in 2'-FL biosynthesis were linked by redox cofactors and energy generation.

Task 1.2. Genome-scale metabolic model (GEM)

An existing GEM of *E. coli* was extended to include the reactions involved in 2'-FL biosynthesis and used to assess the metabolic bottlenecks preventing a higher carbon flux towards 2'-FL production. The GEM was combined to a fermentation model to predict the behavior of *E. coli* during fermentations.

Task 1.3. Identification of bottlenecks

The conceptual metabolic model and the GEM allowed the identification of potential limiting factors hindering 2'-FL production. Based on the model's results, genetic modifications were proposed to the partners to improve 2'-FL biosynthesis further.

Task 1.4. Model verification

The model was validated using data from several existing and new fermentation experiments using different glucose feed rates.

Tasks 1.5. Genetic modifications

The targets proposed to increase 2'-FL production in *E. coli* were discussed with the partners and Glycosyn selected and realized some genetic modifications.

Tasks 1.6. Strain evaluation

The effect of the genetic modifications was evaluated by Glycosyn and FrieslandCampina. The best alternative strain created in this WP1 was then used in WP2 to optimize 2'-FL production.

WP2. Physiological studies

Task 2.1. Medium optimization

Several medium compositions were identified that gave the desired impact on 2'-FL production.

Tasks 2.2. and 2.3. Transcriptome analysis and limited metabolomics

These tasks were moved to WP3.

Task 2.4. Genetic modifications

The results of task 2.1. showed that no genetic modifications were necessary to reach the desired cultivation goals.

Tasks 2.5. Alternative cultivation protocols

Based on the results of task 2.1. new cultivation protocols were set up and tested.

WP3. Stability of expression

Tasks 3.1 to 3.3 could be combined, by co-expressing the 2'-FL genes with green fluorescent protein. The experiments gave some insights in the stability of expression, but also showed some inconsistencies caused by the analytical tools. New experiments with more suitable tools were proposed but could not be tested due to COVID-19 restrictions.

Tasks 2.2 and 2.3. These were not performed because of lab access restrictions due to COVID-19.

Tasks 3.4 and 3.5. (by Glycosyn and FrieslandCampina respectively, outside of the KPI project) have therefore not been performed yet.

WP4. Acetate production

A literature study was conducted to understand acetate formation and its regulation in *E. coli*. We proposed several options to reduce acetate production during fermentations. These include the knock-out of genes involved in acetate formation (*poxB*, *ackA* and *pta*, and if required *eutD* and *tdcD*, the overexpression of the *acs* gene encoding the acetyl-CoA synthetase (ACS) which is responsible for the conversion of acetate into acetyl-CoA using ATP, replacing the PTS system by the GalP-Glk system, and further improving the 2'-FL production capacity.

WP5. Project management and reporting

Every three months all partners were updated on the results obtained in the previous quarter and on the planning on the next quarter.

4.2 Deliverables (meetings and other output, outside of what is listed in 4.3 and 4.4)
Quarterly meetings and progress presentations, a final confidential report
4.3 Communication (lists)
4.3.1 Scientific articles and their DOI (*Digital Object Identifiers)*
n.a.
4.3.2 Reports/articles in journals
n.a.

4.3.3 Other communications (introductory sessions/posters/radio/TV/social media/workshops/exhibitions)
Presentation by Wouter Kuit (FC) at the annual meeting of Microbial Biotechnology, 4 November 2019, Delft.
4.4 Other results: techniques, devices, methods
• New techniques that have the potential to improve 2'-FL production • Identified options to reduce acetate production
4.5 Project website: provide the link to the project website (if available)
n.a.

Final report

5. TRL upon project completion

Technology Readiness Level (TRL) of the technology when completing the project. There are two indicators that differ in the level of detail. If possible, fill in the level of detail. If not possible, fill in the main category.

5.1 Main category *(select one option)*	Industrial research
5.2 Detail category at the start of the project (*number of the category concerned, see appendix for explanation*)	The project entails the improvement of an existing production process (running at TRL9). The TRL level of the improvement started at TRL1
5.3 Detail category at the end of the project	The improved 2'FL production process is at TRL4 The options to decrease acetate production is at TRL2

6 Project status upon completion

Project status *(select one option)*	The project has been completed in accordance with the original scope and all milestones were achieved.

7 Output across the whole project

		Number
7.1	**Number of scientific publications achieved** *Published articles in peer-reviewed journals.*	0
7.1 List	See list below 4.3.1, add articles from previous years (including DOI), if any.	
7.2	**Number of anticipated scientific publications** *Publications expected to be published in a peer-reviewed journal.*	0
7.2 List		
7.3	**Number of non-scientific publications achieved** *Reports, journal articles.*	0
7.3 List	See list below 4.3.2, add publications from previous years, if any.	

7.4	**Number of requested patents** *The number of patents requested on the basis of research within the project.*	0
7.4 List	Provide the DOI for each patent, if available.	
7.5	**Number of licences granted** *The number of licences granted on the basis of research within the project.*	0
7.5 List		
7.6	**Number of prototypes** *The number of developed prototypes on the basis of research within the project.*	0
7.6 List		
7.7	**Number of demonstrators** *The number of developed demonstrators on the basis of research within the project.*	0
7.7 List		
7.8	**Number of spin-offs/spin-outs** *The number of spin-offs and spin-outs resulting from research within the project.*	0
7.8 List		
7.9	**Number of new or improved products/processes/services introduced** *The number of products, processes, and services that were improved or newly developed on the basis of research within the project.*	2
7.9 List	• Modifications (at TRL4) to improve 2'-FL production • Options (at TRL2) to decrease acetate production	

8 Impact

Impact concerns the story of the project: a qualitative description of how the project has contributed to the missions and/or the realisation of economic opportunities. Indicate what will be done with the developed knowledge/tools from the project. Explain the broader contribution of the project to the social challenge, as described in 1.4b. The impact mentioned may relate to topics such as:

- products, concepts, knowledge, etc. applied in practice by the partners, now or in the foreseeable future;
- an interesting example listed as output (section 7);
- insight into preconditions (other than knowledge and innovation) that are necessary to achieve the mission objectives (e.g. funding, regulations, communication, etc.);
- reaching partners and strengthening the networks that have been created;
- connection with practical education and other methods of dissemination.

Provide a link to the website of the project, video, or infographic (if applicable).

Describe the impact of the project, *provide a link to the project website, a video, or infographic (if applicable).*
The project enabled a good collaboration between WUR, Glycosyn and FrieslandCampina.

WUR created a genome-scale metabolic model and fermentation model that can be used to predict 2'-FL production using different strains and/or different cultivation conditions. The model has also been applied in other *E. coli* projects.

The project increased knowledge in *E. coli* metabolism and insights on how to improve 2-FL production and increased knowledge of acetate production by *E. coli*

FrieslandCampina obtained increased understanding and control of the fermentation process. The obtained knowledge is used in the strain development work, which resulted in a new strain with higher yield for which the preparation and implementation stages for industrial scale is planned.

The project results are input that supports the availability of human milk oligosaccharide 2'FL for infant nutrition. Increased control and higher yield will make the ingredient cheaper and it improves FrieslandCampina's position in the competitive field

Appendix 2 TRL categories

The detail categories are:
TRL 1 – basic principles have been observed and reported
TRL 2 – technological concept and/or application has been formulated
TRL 3 – critical function or characteristic has been analytically and experimentally proven
TRL 4 – component or experimental model has been validated in a laboratory environment
TRL 5 – component or experimental model has been validated in a relevant environment
TRL 6 – system/sub-system model or prototype has been demonstrated in a relevant environment
TRL 7 – prototype of the system has been demonstrated in an operational environment
TRL 8 – the actual system has been completed and has been qualified through testing and demonstration
TRL 9 – the actual system has been validated by a successful operational company