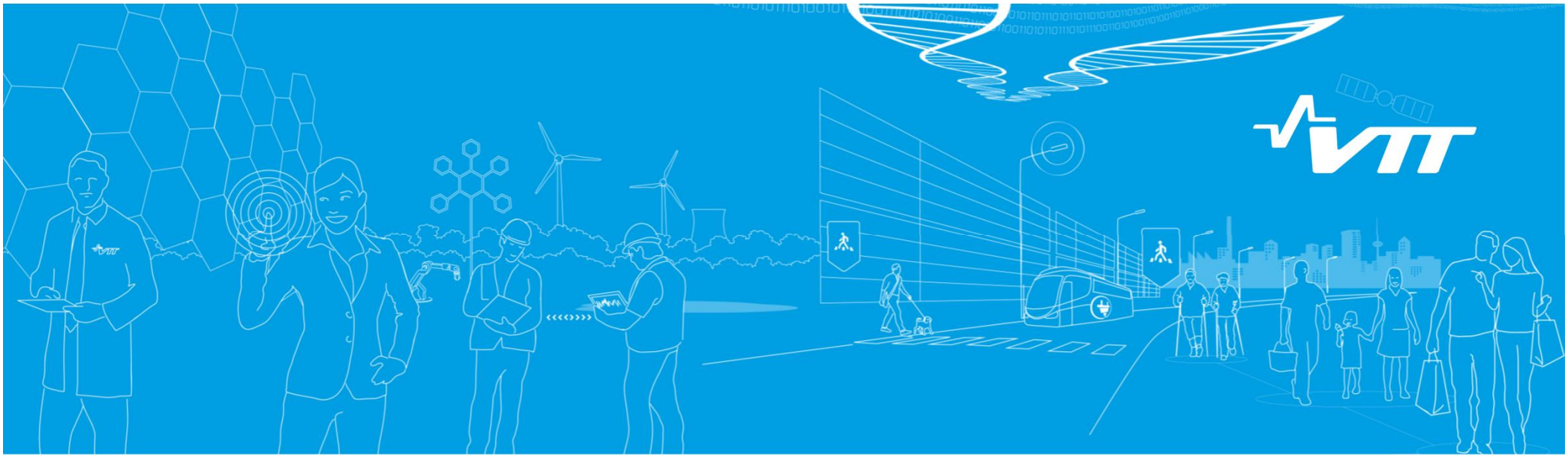

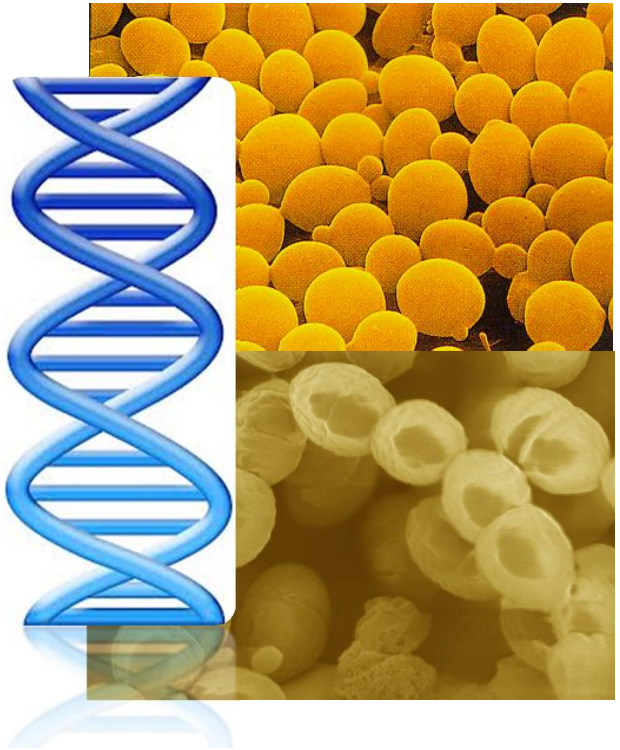

Control of *in vivo* polymerisation by synthetic biology approaches

Opening Seminar of Synthetic Biology Research Programme – FinSynBio

23rd of January 2014, Helsinki

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Control of *in vivo* polymerisation by synthetic biology approaches (SynbioPol)

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Bio-based polymers

There is a growing interest in utilising bio- and bio-based polymers in various applications.

- Biopolymers from monomeric building blocks
  - Monomers such as glycolic acid, lactic acid, succinic acid, 1,3-propanediol, or 1,4-butanediol can be produced from renewable resources by microbial fermentation and polymerised with traditional chemistry.
- Natural polymers /Polymerisation by nature
  - Polymerisation *in vivo*, generating *e.g.* cellulose, xylan, glucan, chitin, hyaluronan and polyhydroxyalkanoates has several benefits.
  - The fermentative production of polymers is a mild process, and does not require extremely pure monomers, for avoiding side reactions and poisoning of the catalyst during polymerisation. The fermentative polymerisation favours synthesis of ultra-high molecular weight copolymers, since there is no mixing problem that is generally encountered during chemical polymer synthesis due to the high viscosity that increases as the reaction proceeds (Park *et al.,* 2012).

Bioprocess for biopolymer production
in vivo vs. *in vitro* polymerisation

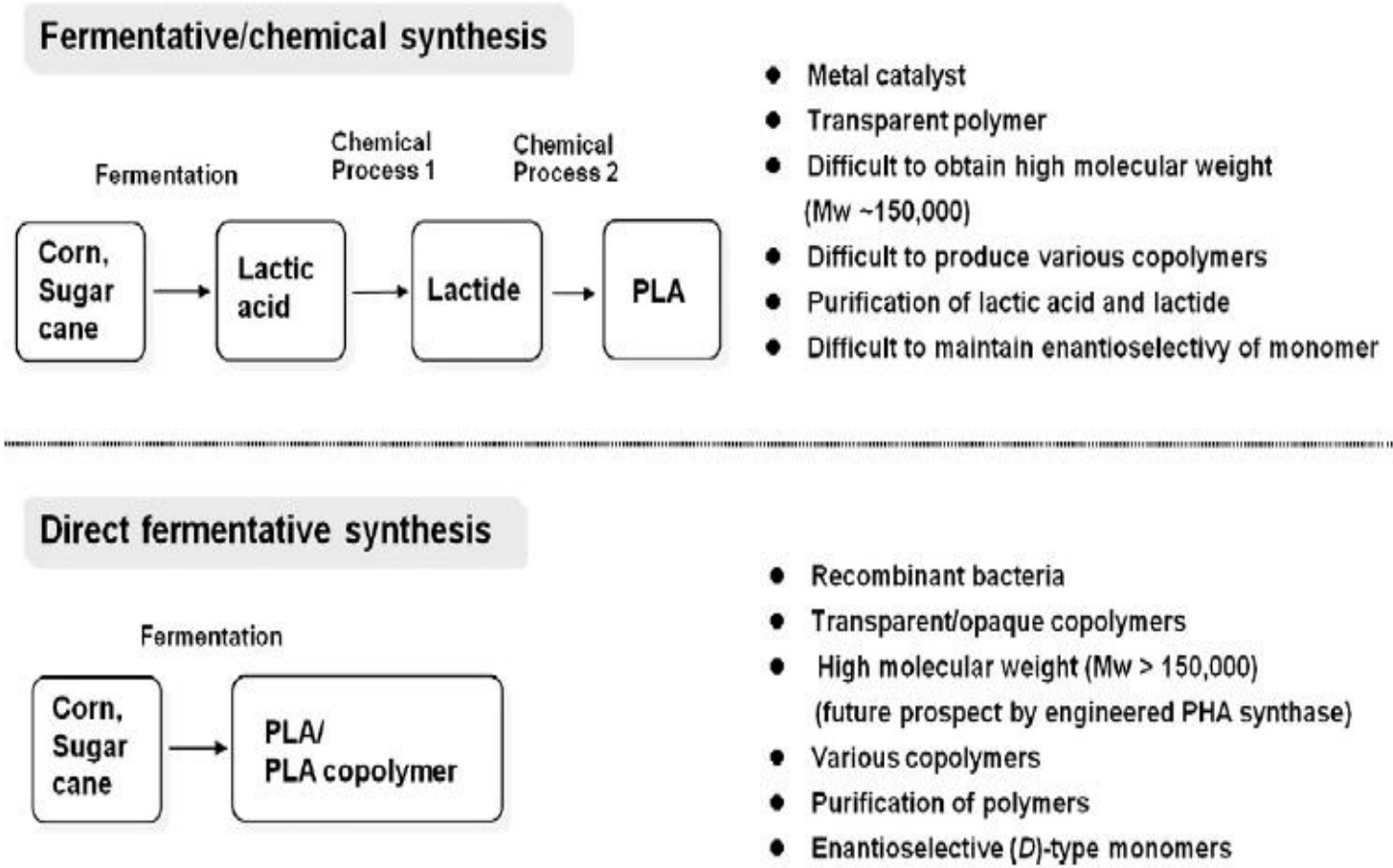


Fig. 5. Comparison of direct fermentative synthesis of PLA and typical bio-chemo hybrid synthesis of PLA. (*D*)-lactic acid is used for PLA synthesis in microorganisms, whereas (*L*)-lactic acid is mainly used for PLA synthesis using a bio-chemo process.

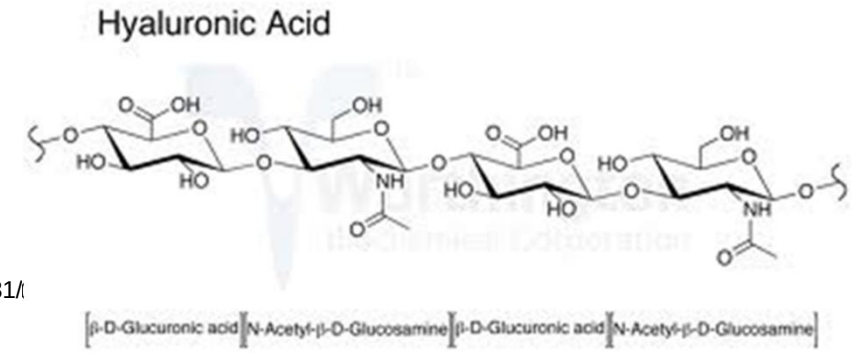
Objectives of SynbioPol

Optimisation of *in vivo* polymerisation of two study cases, production of hyaluronic acid (HA) & polyhydroxyalkanoate (PHA) using synthetic biology (scaffolding) to enhance spatial coordination of the synthesis reactions in *S. cerevisiae & L. lactis*

- **Synthetic biology** aims to engineer novel reactions, regulatory circuits, and organisms which can be assembled from standardised biological parts in a rational way for any desired target.
- **Spatial coordination of reactions:** Subcellular co-localisation of pathway enzymes may increase the local concentration of a metabolite and thus favour the nearest reactions. Spatial proximity may be achieved by compartmentalisation, as in bacterial cellulosomes or reactions targeted to peroxisomes or vacuoles. Enzyme activities may occur in complexes, such as with polyketide synthases. Synthetic scaffolds (Dueber *et al.,* 2009) may be built to target enzymes into spatial proximity in desired ratios for enabling optimal flow of substrates and products.

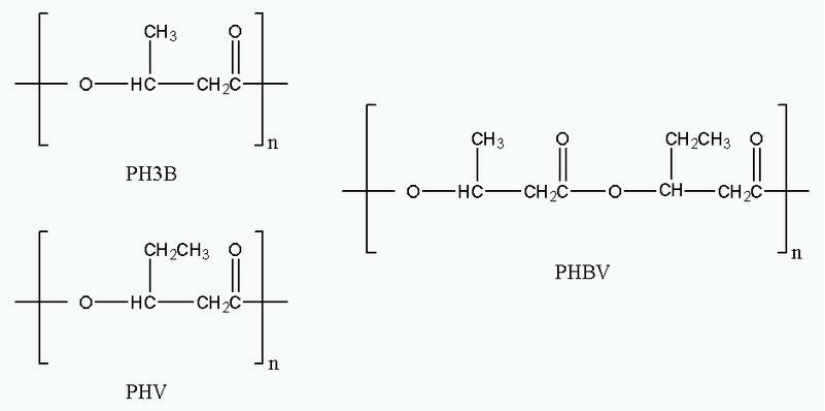
Hyaluronic acid (HA)

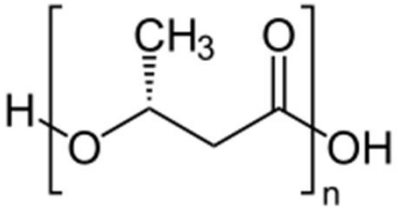
- Hyaluronic acid (HA), also called hyaluronan, a linear glycosaminoglycan polysaccharide composed of repeating disaccharide units of alternating D-glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc), is a mucopolysaccharide occurring naturally throughout the human body.
- HA has high water-holding capacity, viscoelasticity and biocompatibility, and finds wide applications in biomedical, food, healthcare and cosmetic fields; skin moisturisers, osteoarthritis treatment, ophthalmic surgery, adhesion prevention after abdominal surgery, and wound healing
- HA has traditionally been extracted from animal tissues such as synovial fluid, rooster combs, cartilage, vitreous humour and umbilical cords, and by native producers such as *Streptococcus zooepidemicus*.
- The HA pathway has been metabolically engineered in many bacterial systems; *B. subtilis*, *E. coli* and *L. lactis,* and in yeast *S. cerevisiae*
- However, **the yield and molecular weight of HA** obtained in all these studies is **lower** than that of the natural producer *S. zooepidemicus*.

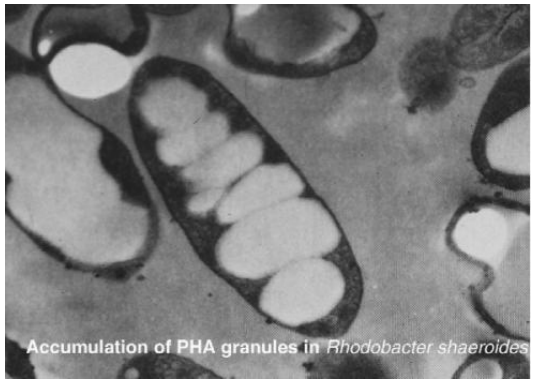


Polyhydroxyalkanoates (PHA)

- Many bacteria accumulate PHAs in their cytoplasm to store carbon and energy, as the native PHA producer *Ralstonia utropha*. Some eukaryotes accumulate small amounts of PHA and yeast, including *S. cerevisiae* have been engineered to produce PHA.
- Biosynthesis of PHA occurs in two steps: generation of hydroxyacyl-CoAs (HA-CoAs) followed by the polymerisation of the HA-CoAs by PHA synthase into PHAs. Various compounds such as sugars, lipids, alkanes, alkenes and alkanoic acids can be converted into PHAs and more than 150 different HA-CoAs have been identified as PHA monomers.
- Recently, several studies describe the incorporation of 2-hydroxyacid monomers such as glycolate, lactate, and 2-hydroxybutyrate into the PHA copolymers by natural and engineered PHA synthases.
- Although already in commercial scale, PHA production, especially its copolymers, have challenges (substrate specificity & activity of the PHA synthase towards non-native substrates, molecular weight of the co-polymer)







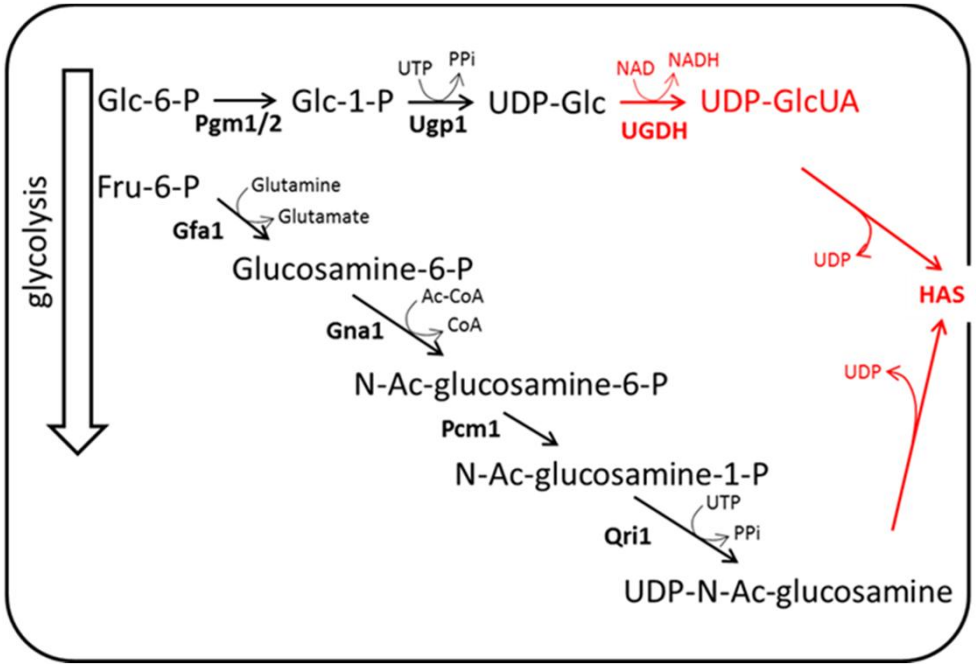
Protein scaffolds; spatial coordination of reactions

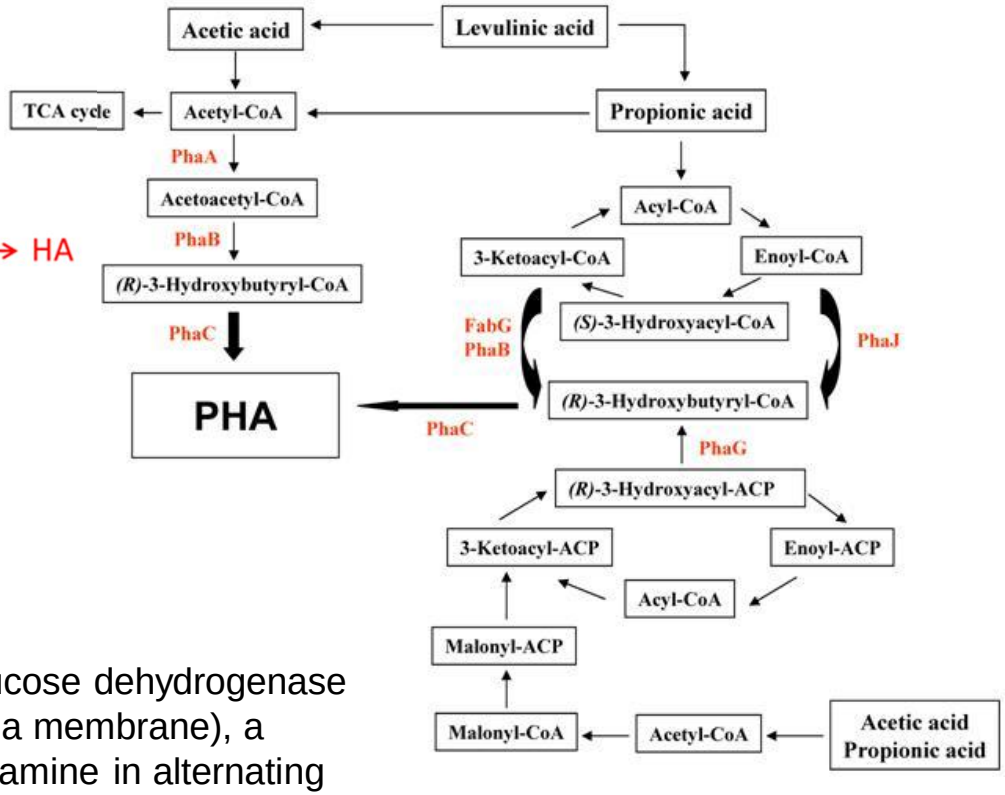
- Protein scaffolds have been built by fusing three eukaryotic protein-protein interaction domains (PDZ-domain from mouse adaptor protein α-syntrophin, SH3-domain from mouse adaptor protein Crk, and GBD-domain from rat actin polymerisation switch protein) and co-expressing the desired proteins as fusions with their cognate binding domains.

- The strategy allows for the localisation and stoichiometric control of a limited number of proteins and has been successfully applied to improve production of resveratrol in *S. cerevisiae*, and glucarate, hydrogen, and flux to mevalonate pathways in *E. coli.* (Dueber *et al.* 2009, Moon *et al.* 2010, Wang *et al.* 2009 & 2012, Agapakis *et al.* 2010)

Research Focus

- Development of methodologies for improved production of high molecular weight bio-based co-polymers by engineered *Lactococcus lactis* and *Saccharomyces cerevisiae*. Two study cases addressed; microbial production of hyaluronic acid (HA) and polyhydroxyalkanoates (PHA)

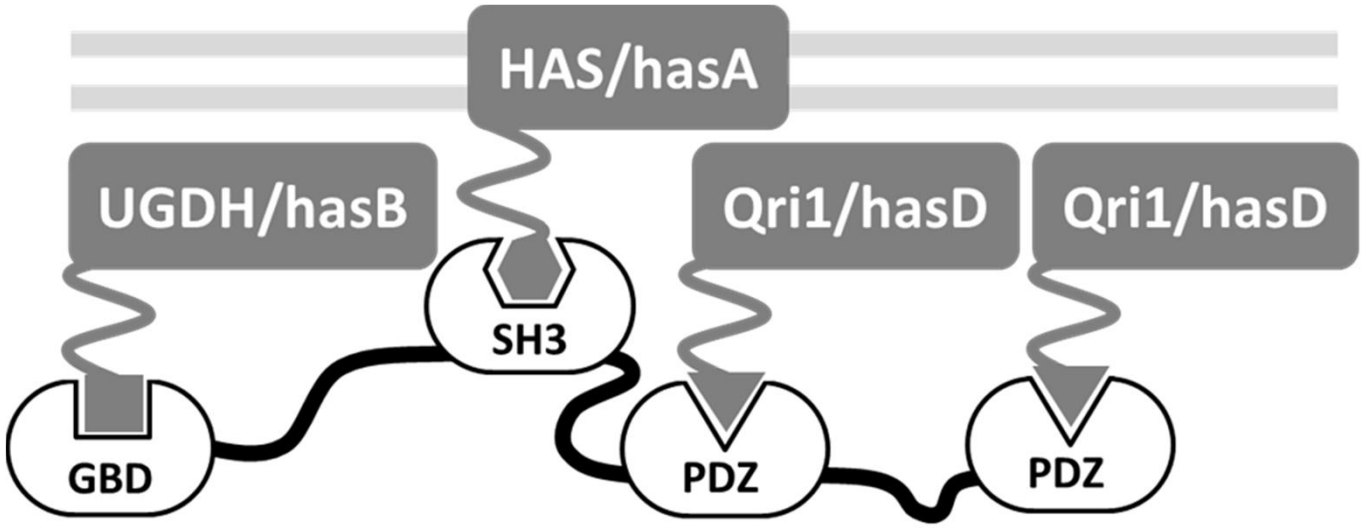




UPD-glucose is converted to UDP-glucuronic acid by UDP-glucose dehydrogenase (UGDH) and NAD^+. The hyaluronan synthase (HAS; on plasma membrane), a glycosyl-transferase adds glucuronic acid and N-acetyl-glucosamine in alternating positions. The HA chain is formed at the external part of the plasma membrane creating a pericellular HA coat. The production of HA naturally competes for glucose-6-phosphate and fructose-6-phosphate with other cellular pathways.

Scaffolding strategy of HA biosynthesis

- The enzyme complex formed by the scaffolding technology will contain three final enzymes of the HA biosynthesis (UDP-glucose pyrophosphorylase, UDP-N-acetyl-glucosamine pyrophosphorylase, hyaluronan synthase).



The synthetic protein scaffold (composed from the protein-protein interaction domains – GBD, SH3, and PDZ) mediates the co-localisation of the key enzymes (fused with specific binding motives) at the site of the HA biosynthesis. The integral membrane enzyme, hyaluronan synthase (HAS), binds the SH3 domain of the synthetic adaptor protein (scaffold), the interaction leading to recruitment of the other two enzymes producing the intermediates required for the HAS reaction. This enzyme assembly facilitates production and channelling of the intermediates, providing increased local concentration and better accessibility for the HA synthase. In addition, the modular structure of the scaffold offers variation in ratio of the enzyme-binding domains for achieving an optimal enzymes stoichiometry (1:1:2 shown).

Research Objectives & Methods / 1

- **Synthetic scaffolds** for enhancing the spatial proximity of the polymer precursors. The concepts of channelling of the pathway intermediates, and increased sub-cellular/local concentrations of the key precursors, to enhance the production, will be tested by the use of synthetic protein adapters (scaffolds) for co-localisation of the crucial enzymatic activities to optimise the stoichiometry of the production pathway components. The localisation of HA synthesis in the plasma membrane creates additional challenges for the scaffold design.

- **Metabolic networks modelling** (such as flux analysis) and **Metabolic engineering** (such as selected gene deletions and over-expressions). Particular emphasis will be put on the control of synchronised synthesis of the two metabolites (the monomers in the co-polymers) produced in two different metabolic pathways (e.g. UDP-glucuronate and UDP-N-acetyl-glucosamine). When available standardised promoter libraries, will be used to drive the optimal expression of the genes encoding the key reactions, in order to achieve correct timing of synthesis of the metabolites in context of the physiological state of the production hosts.

Research Objectives & Methods / 2

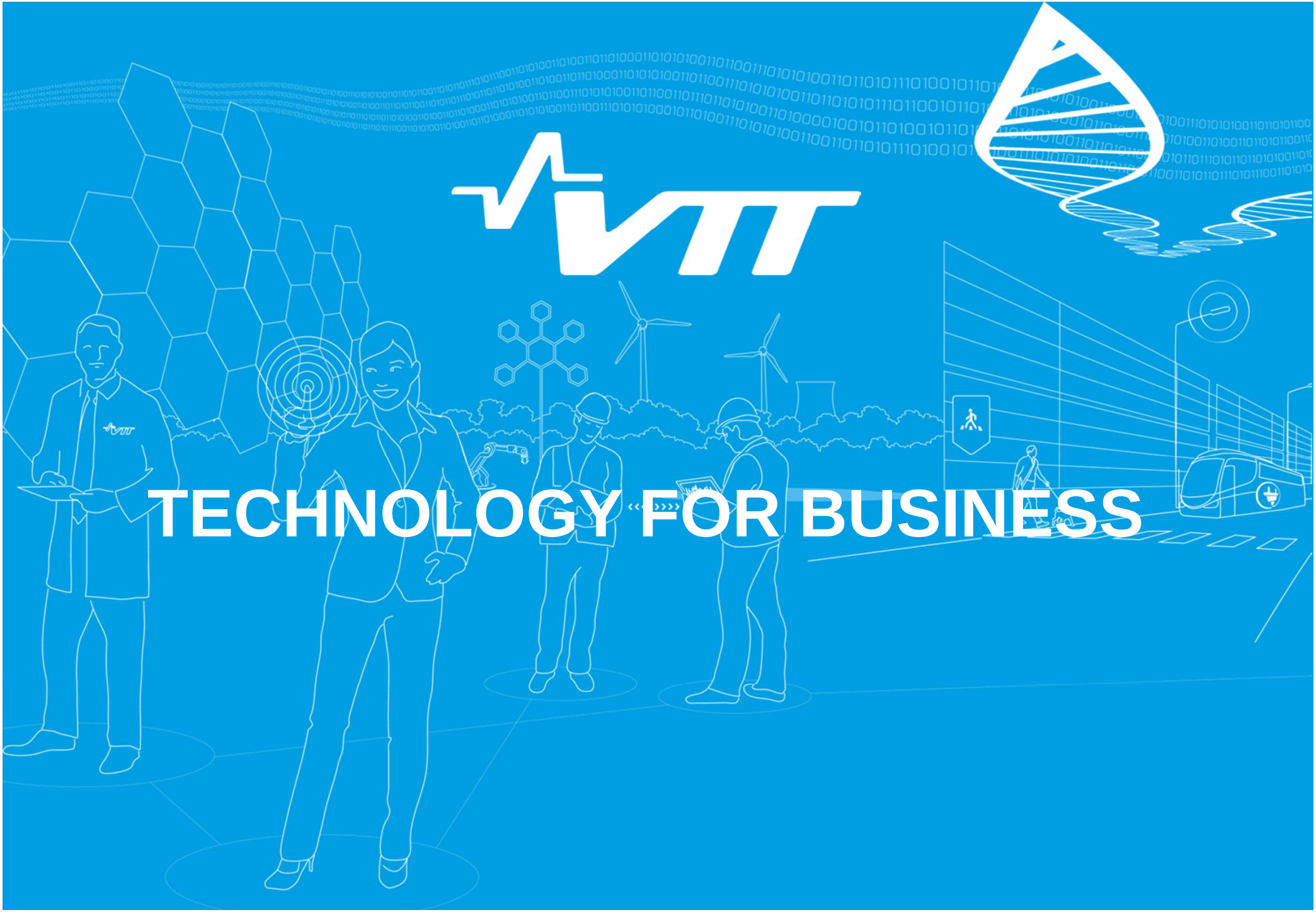
- Development and improvement of the **Bioprocess techniques**. As the intermediates of the biopolymers are also essential precursors for numerous biological processes of the host organisms, the fine-tuning of balance between the biomass formation and the polymer production is an important issue. It has been shown that fermentation conditions including pH, agitation speed, aeration rate, and shear stress have significant influence on the quality and quantity of the produced polymer. In addition, the metabolic fluxes are significantly influenced by the cultivation conditions. On-line bioprocess monitoring and control will be used in combination with the above described approaches in synthetic biology and metabolic engineering to improve high MW polymers production.

- Metabolic network modelling & target identification (IITM)
- Metabolic engineering of *L. lactis* & *S. cerevis*iae for production of polymers (VTT, IITM)
  - Bench mark strains & synchronising precursor production for HA
- Synthetic scaffolds in *L. lactis* & *S. cerevisiae* for production of polymers (VTT, IITM)
  - Introducing scaffold structures for HA & PHA production
- Bioprocess studies for enhancing productivity & molecular weight of HA in metabolically engineered *L. lactis* & *S. cerevisiae* (IITM, VTT)
  - Bioprocess modelling & process optimisation, on-line monitoring & process control

Outcome

- The project explores (through two case studies) the use protein scaffolds for linking pathway enzymes with the objective of enhancing and controlling the production of high MW co-polymers. A combination of synthetic biology and process engineering approaches will be used to achieve this objective.

- The success of this project will allow the approach to be generically used for production of other co-polymers and also adopted in different host organisms.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