Assessment Framework Fermentation

Evaluating Investment Opportunities in Fermentation-based Technologies

Wageningen Food & Biobased Research
Expertise Cluster Microbial Cell Factories

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Dear Reader,

The global protein transition is essential for creating a sustainable and resilient food system. Animal-based food systems are among the largest contributors to biodiversity loss and climate change. Agriculture accounts for 30 percent of global anthropogenic greenhouse gas emissions, with animal products responsible for nearly 60 percent of these emissions. Moreover, agriculture drives more biodiversity loss than any other sector, while the bio-industry places significant pressure on animal welfare. With the global population projected to reach 10 billion by 2050, the need for sustainable and scalable food solutions is more urgent than ever.

The protein transition offers a transformative opportunity to address these challenges by shifting to innovative, low-impact, and sustainable protein sources. The Netherlands, with its strong agrifood ecosystem and rapidly expanding alternative protein sector, is uniquely positioned to lead this transformation. The alternative protein market in the Netherlands, valued at € 346 million in 2022, is expected to grow to over € 10 billion by 2030 (Foodvalley), highlighting the tremendous opportunity for impact and investment.

One particularly promising submarket within this transition is alternative dairy. This segment offers scalable, sustainable, and low-impact protein solutions, reducing greenhouse gas emissions, resource use, and reliance on traditional dairy farming. It also addresses growing consumer demand for lactose-free, ethical, and health-conscious products. The global plant-based dairy market, valued at $ 28 billion in 2023, is projected to grow to $ 91 billion by 2032 (Fortune Business Insights). Notably, several innovative Dutch companies are driving growth and offering substantial opportunities for further innovation in this space.

Despite its potential, the sector faces notable challenges that impact investments, including high initial capital requirements, technologically demanding and lengthy scaling processes, and navigating strict regulatory frameworks. To overcome these barriers, we have developed a benchmarking framework in collaboration with Wageningen University & Research. This tool provides investors with actionable insights to evaluate technologies and make strategic decisions. Additionally, we are fostering a co-investor network to enhance collaboration and knowledge sharing — critical for advancing this capital-intensive market.

We firmly believe that reducing financial barriers and promoting investments in high-impact markets will accelerate the global protein transition and contribute to a more sustainable and secure food system. We invite you to join us in shaping this transformation.

Sincerely,

Invest-NL Agrifood

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Our understanding

Vision Assessment Framework

The **vision of Invest-NL** is that it is of high importance that investors are well informed on the potential and challenges in the (precision) fermentation space.

This document assists investors in making the right choices and guide their prospect into addressing the most prominent challenges in industrialization or commercialization of there specific processes.

Assignment

The **framework** is setup with the following goals in mind:

- To develop general benchmarking guidelines for assessing investments in companies involved in general fermentation, biomass fermentation, and precision fermentation with a focus on alternative protein.
- To facilitate investors in asking such companies targeted questions to gauge the viability of their proposition.

Deliverables

The deliverable is a slide deck that provides the following:

- An **introduction** to the topic.
- **Background information** on relevant technical concepts.
- **Questions** investors can pose to investment solicitors to evaluate potential technical and general challenges.

The framework

Scope & limitations

Scope

The Assessment Framework focuses on the technical aspects of fermentation-based processes and aims to assist investors with:

- Initial evaluations of opportunities in general, biomass, and precision fermentation.
- Evaluating scalability and industrialization risks in the fermentation space.

Limitations

The Framework does not:

- Replace a due diligence approach conducted by experts.
- Include financial analysis or specific investment recommendations.
- Provide a full lifecycle analysis (LCA) or environmental impact study.

Disclaimer

- This document is for informational purposes only and does not constitute financial or investment advice.
- Users are encouraged to seek professional guidance for decision-making based on the framework’s insights.
- The content aims to help identify technical challenges and risks but is not exhaustive

Table of contents

1. Introduction to fermentation processes

Definition of fermentation

Process of growing a microorganism on a feedstock to convert the feedstock into desired products.

Scope of the assessment

Production of plant- and/or microbe-based meat and dairy protein alternatives using fermentation.

Types of fermentation in this framework:

General | Biomass | Precision

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Fermentation types: General fermentation

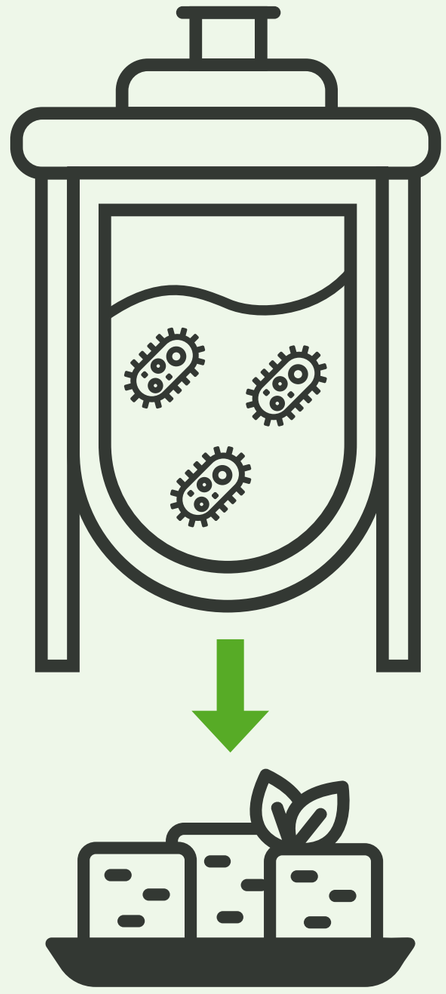
No new protein is produced, but specific properties of an existing (plant-based) source of protein are modified by microorganisms.

Taste, texture and/or nutritional qualities can be modified. For example:

- Acids and alcohols are produced from the sugars that are converted;
- Macronutrients (e.g. amino acids, sugars) are converted to specific flavor components;
- Proteins and polysaccharides are (partially) broken down, which can alter texture or impact digestibility.

Microbial cells remain present in the product after fermentation. Food-grade strains are used. These strains are selected for their capacity to perform the desired conversions. Fermentation processes may involve either single or multiple microorganism types. Examples are tempeh, fermented tofu, miso, soy sauce, sauerkraut.

Plant based protein source



- **Plant-derived proteins: no conversion needed**
- **Low CAPEX/OPEX vs. other fermentation types**
- **Flavor/texture not identical dairy/meat.**

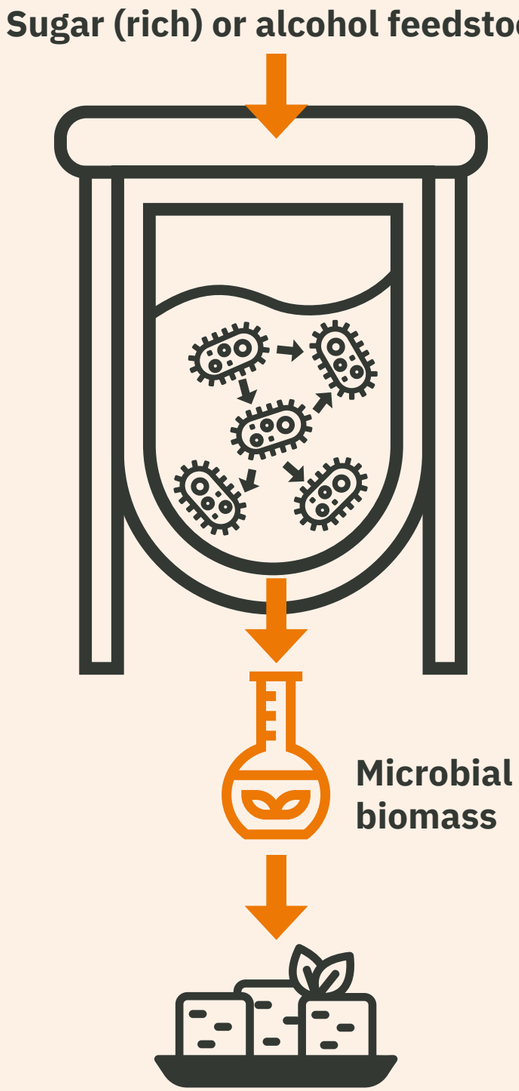
Fermentation types: Fermentation for biomass production

The purpose is producing microbial cells (biomass) as a source of protein: the microorganism itself is the product.

The fermentation starts with a small amount of microbial cells, and by feeding those cells sugars and nutrients the number of cells (the total biomass) increases. Since microbial cells contain a lot of protein (also termed: single-cell protein), this biomass can then be used as alternative protein product.

E.g. Quorn™ is biomass of the fungus Fusarium venenatum, which after fermentation has been further (mechanically) processed to alter textural properties.

Food-grade strains are used. These are selected for the natural presence of favorable nutritional properties or texture/flavor, as well as for their capacity to grow to high cell densities.



- **Straight forward process: sugar > biomass**
- **High nutritional value from whole cell protein**
- **Limited room for modulation of flavor/texture**

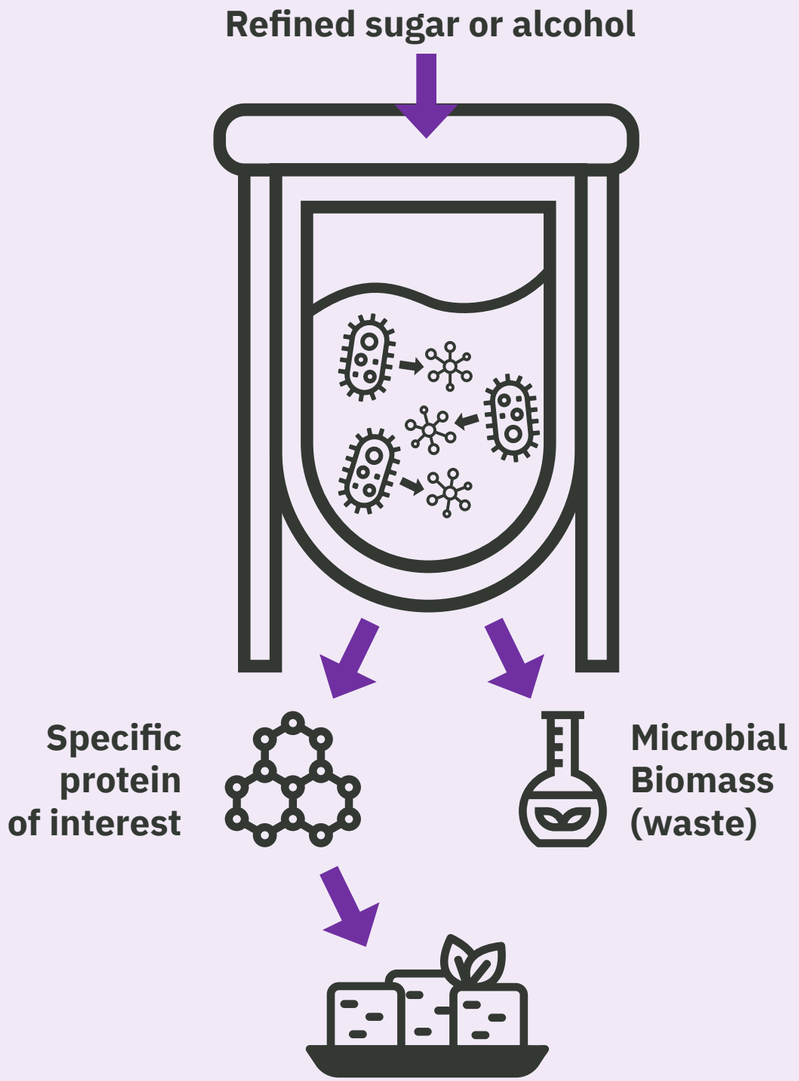
Fermentation types: Precision fermentation

The term precision fermentation has been described by the Precision Fermentation Alliance (PFA) and Food Fermentation Europe (FFE) as follows:

“Precision fermentation combines the process of traditional fermentation with the latest advances in biotechnology to efficiently produce a compound of interest, such as a protein, flavor molecule, vitamin, pigment, or fat. A specific DNA sequence is inserted into a microorganism to give it instructions to produce the desired molecule when fermented. These molecular sequences are derived from digitized databases rather than taken directly from the relevant animals or plants. At the end of the Fermentation process, the resulting compounds are filtered out, separating them from the microorganisms that produced them.”

In other words: the genetically modified microorganism (GMO) converts a substrate into a very specific component, such as a specific protein. During fermentation not only this protein of interest, but also microbial cells and other (by)products are produced. These can represent a significant portion of the total fermentation product and will need to be removed afterwards. In addition to the fermentation itself, ensuring protein purity, quality and functionality are major challenges. An example of a protein produced by precision fermentation is leghemoglobin, which mimics meat flavor in the Impossible™ Burger.

- **Proteins identical to animal-based**
- **GMOs as side-product**
- **Large CAPEX to reach high purity**



2. Framework assessment

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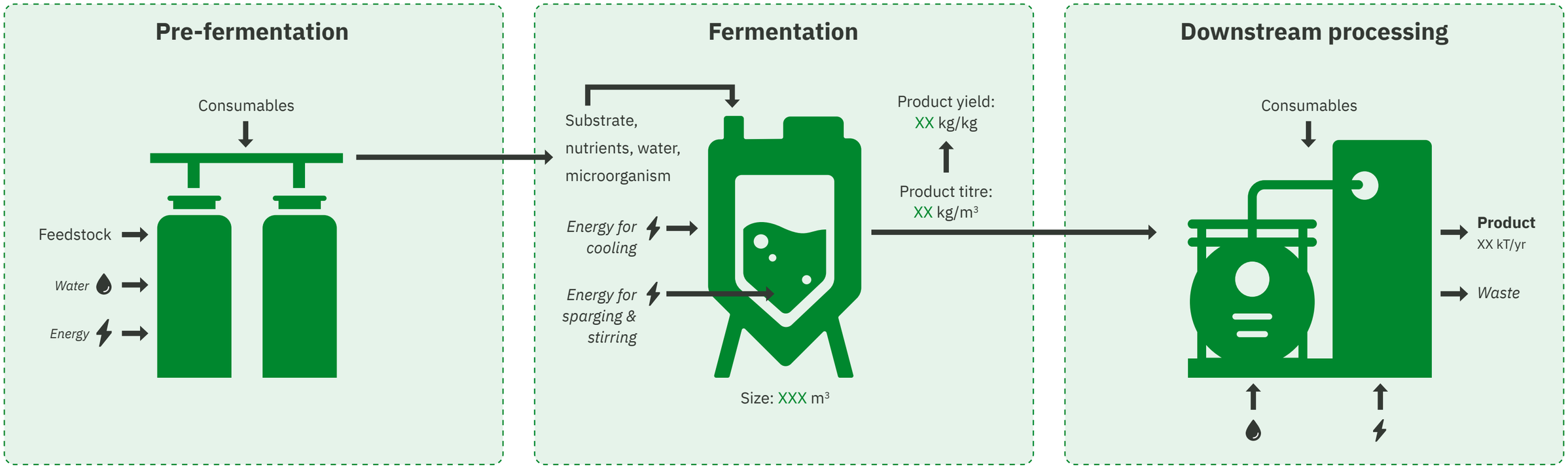
Fermentation stages



Before, during, and after fermentation

In general, a fermentation-based commercial process can be divided into three stages:

Pre-fermentation: preparing all materials that are required for the actual Fermentation process (also referred to as ‘upstream processing’ or USP).

Fermentation: the conversion of the substrate into the product by means of microbial activity.

Downstream processing: separating the product from other materials (waste) and verification of product quality parameters (abbreviated as DSP).

In this framework, these three stages will be used as ‘containers’ for sub-themes that may require more attention. Especially for fermentation and downstream processing, there can be different focus points among the three defined fermentation types (general, bio-mass, precision) for de-risking the industrial process development.

Framework assessment

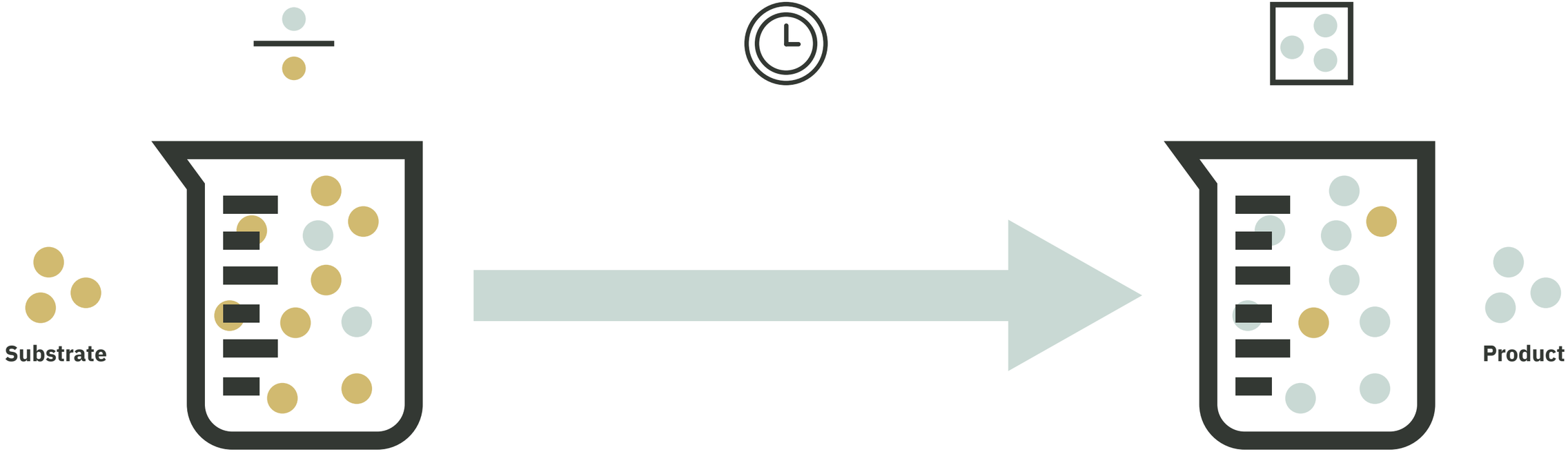
Trade-offs in Titer, Rate and Yield (TRY)

Achieving optimal performance in fermentation is centered on maximizing Titer (product concentration), Rate (production speed), and Yield (efficiency of resource conversion) or TRY. These KPI’s are critical benchmarks for success. Enhancing a single KPI may lead to reduced performance in another. Therefore, it is crucial to carefully balance these trade-offs.

Yield
product produced per
substrate consumed

Rate
Increase / decrease
in time

Titer
product concentration



Start with the end in mind

A realistic targeted annual production volume and product application is essential for determination of feedstock and reactor volumes and the requirements for product purity, stability and functionality.

Lab-scale: <1% of final scale

Small-scale cultures

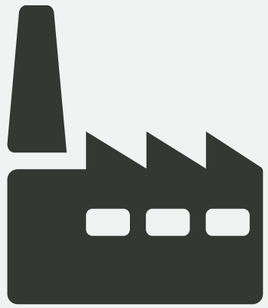
Shake-flask cultures

Lab-bench bioreactors

Pilot-scale: 1-10% of final scale

Pilot/Full-scale bioreactors

Full-scale: 100% of final scale



Industrial production

3. Overview slides

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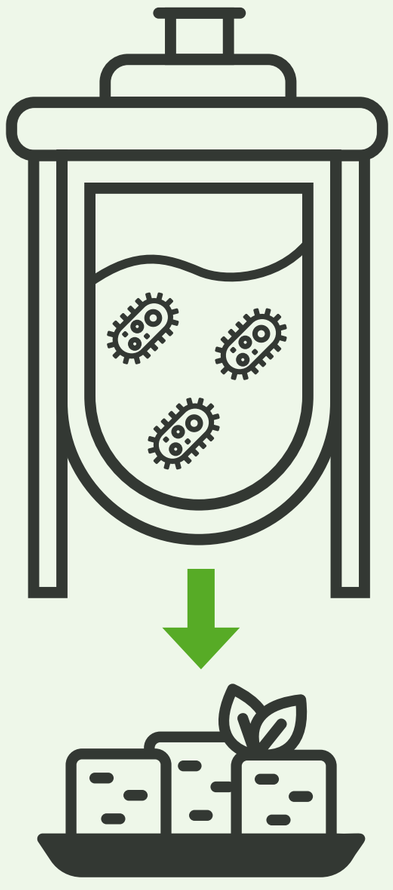
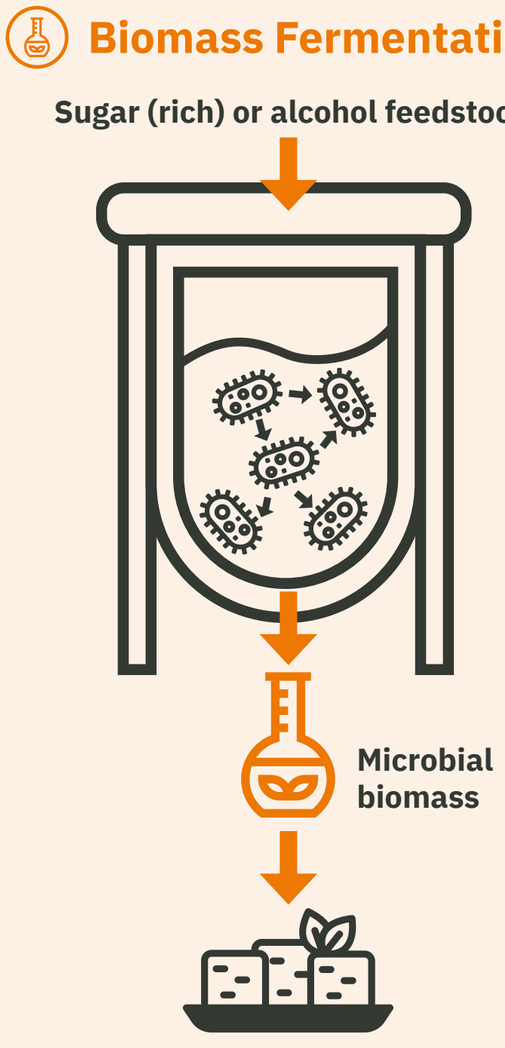
Click a fermentation type to move to the overview

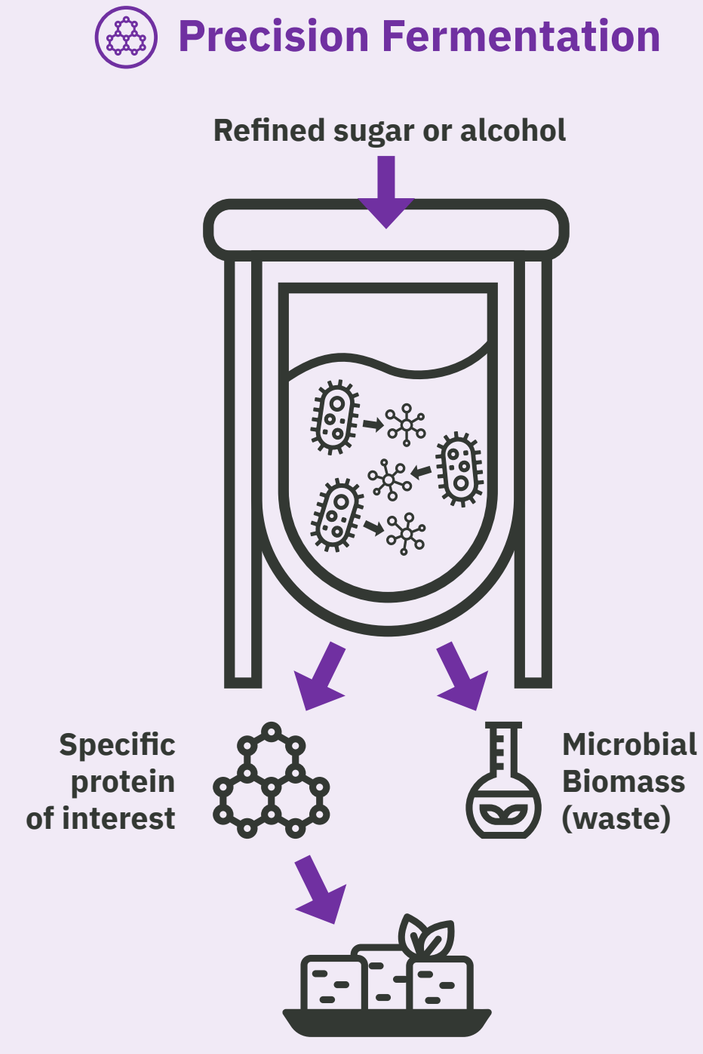
General Fermentation



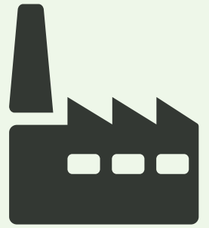
Biomass Fermentation



Precision Fermentation



Key KPI’s, risks, and scale-up considerations in General Fermentation



Lab-scale production → **Pilot/Demo-scale production** → **Industrial production**

Target output:
5 - 350 ton/M3/annum

Typical KPI’s:
fermentation titer: maximal
fermentation yield: maximal

Required at full scale:
- Consistent flavor/texture
- Robust cultivation method

Themes with most important risks:

Fermentation product quality
IP and competitive position
Strain selection
Scalability challenges
Fermentation modes
Feedstock considerations
Sterilization
Waste handling
Regulatory compliance
Aerobic/anaerobic
R&D Infrastructure
Process monitoring & control

Development stage

Key KPI’s, risks, and scale-up considerations in **Biomass Fermentation**

Lab-scale production → **Pilot/Demo-scale production** → **Industrial production**

Themes with most important risks:

- Fermentation product quality
- Feedstock considerations
- Scalability challenges
- Strain selection
- Process monitoring & control
- Downstream processing
- Sterilization
- Titer, rate, yield
- Fermenter aeration
- Pre-treatments
- Waste handling
- Regulatory compliance
- IP and competitive position
- Fermentation modes
- R&D Infrastructure

Development stage

Target output:
2,5-25 ton/M3/annum

Typical KPI’s:
Biomass titer: >100 g/L
Biomass yield: 0.3-0.5 g/g

Required at full scale:
- Large fermenter capacity
- High biomass yield
- Robust cultivation method
- No undesired host-derived impurities (flavors/toxins)

Key KPI’s, risks, and scale-up considerations in Precision Fermentation

Lab-scale production → Pilot/Demo-scale production → Industrial production

Themes with most important risks:

Titer, rate, yield	Host strain R&D	Waste handling		
Protein functionality	IP and competitive position	Downstream processing	Quality control	Sterilization
Host strain selection	Protein stability	Scalability challenges	Fermentation modes	Feedstock availability
Strain construction	Regulatory compliance	R&D Infrastructure	Fermenter aeration	Process monitoring & control

Development stage

Target output:
0.5-2.5 ton/M3/annum

Typical KPI’s:
fermentation titer: 1-20 g/L
fermentation yield:
0.005-0.05 g/g

Required at full scale

- Large fermenter capacity
- High product titer
- Optimal DSP
- Full product functionality

4. Pre-fermentation process

Fermentation processes require nutrient inputs for biomass and product formation. These nutrients can come from pure sources (e.g., sugar, ammonia, minerals) or from more cost-effective and environmentally friendly side-streams/co-products. Side-streams often need pre-treatment due to variability in composition and structure, which can impact fermentation outcomes. Comprehensive testing of these side-streams is essential to ensure consistent quality and suitability for industrial applications.

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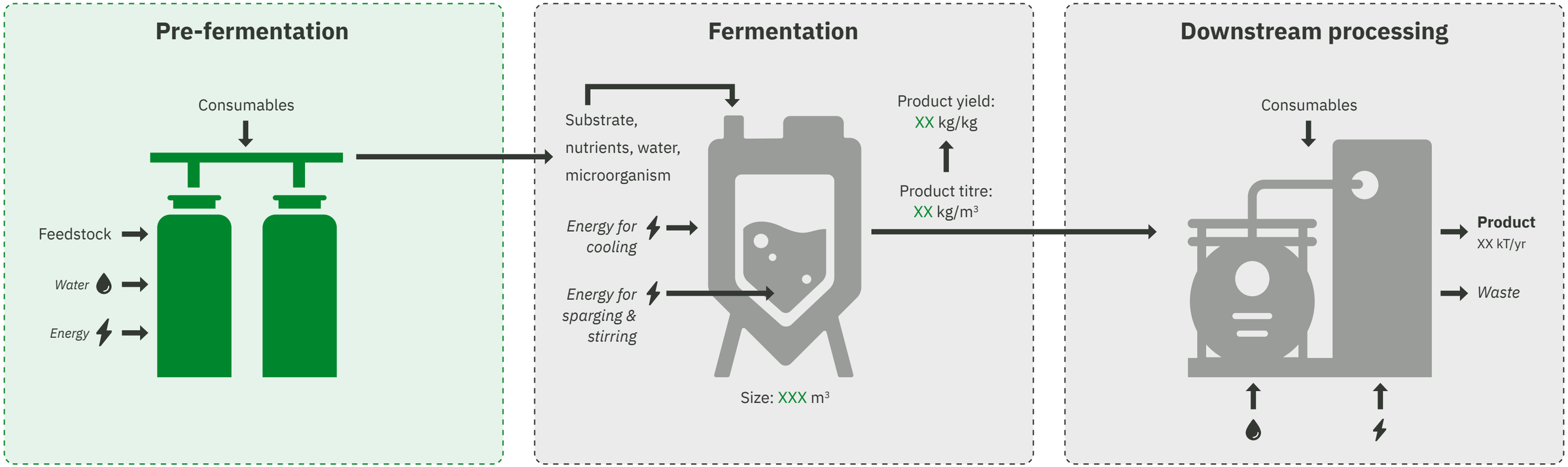
4. Pre-fermentation process



Feedstock considerations

The use of **pure ingredients** ensures highly reproducible processes. This is not always feasible from a cost, environmental or sourcing perspective.

As a cost-effective alternative, **low-cost food grade side streams** may be utilized. When using such side-streams, it is very important to consider the following aspects.

Considerations for side stream usage

Availability:

- Ideally readily available near the production site, year-round.
- Feedstock should be stable, easily transportable, and it should be possible to source multiple times a year to minimize excessive storage needs.

Consistency:

- Seasonal fluctuations and/or production location can impact side stream composition.
- Validation of stable storage across seasons and sourcing locations is essential.

Compatibility:

- Side streams generally do not consist of optimal levels of phosphate, nitrogen, and carbon for microbial growth. Adjusting nutrient composition of the feedstock is vital for maximizing microbial growth.

Inert Material:

- Side streams contain fractions that are not used by microorganisms and therefore require separation from the product. Removal of inert material prior to fermentation can be considered as alternative.

Relevant Questions

- What is the available volume of the feedstock/side-stream?
- Is the feedstock/side-stream available throughout the year or does substrate availability show seasonal variation?
- Can the feedstock/side-stream be stored for longer periods?
- Are supply contracts or pricing discussions established?
- Is the material available at the location of the industrial process or is transport required?

Biomass or Precision Fermentation

- Does the side-stream composition allow for full consumption of all nutrients during fermentation?

Pre-treatments

Pure ingredients can typically be readily dissolved readily in solution to create a growth medium for the fermentation. In contrast, side-streams generally require additional treatment prior to use in a bioreactor, given their more complex composition and structure. Side-streams can fluctuate significantly in quality and composition throughout the year, underscoring the importance of comprehensive testing.

Treatments

Size reduction: Side streams can consist of larger materials that are harder to transport within a process, or in which nutrients are not easily available for microorganisms.

- Milling or crushing into smaller particles is required, for which various approaches (dry or in solution) are available.

Releasing nutrients: Important nutrients (such as sugars) are often locked within polysaccharides or complex structures within side-streams

- Enzymatic, acidic, or heat treatments are essential to release these nutrients and making them accessible for microorganisms.

Detoxification: Side-streams may contain compounds that inhibit microbial activity (kill or slow down microorganisms).

- Detoxification is required before fermentation, and can be achieved through adsorption, chromatography, or extraction methods.

Relevant Questions

- Does the side-stream require pre-treatment?
- Has the pre-treatment process been fully tested in combination with the rest of the process?
- What are the costs for sugar release, if this is a required step?
- Does the substrate contain components that negatively affect KPI's?

Sterilization

Within a Fermentation process, absolute control over which microorganism(s) is or are active is essential. Contamination with non-productive strain should be avoided, as it may lead to a strong reduction in productivity or complete failure of the process. Virtually all feedstocks contain microorganisms that could compromise fermentation.

Therefore, prior to starting the fermentation process, sterilization of the feedstock or nutrients and the process equipment is required.

Sterilization Treatments

Heat treatment is the most common method for sterilization of substrates:

1. Pasteurization: used for short processes with duration shorter than 12 hours as this does not inactivate all contaminating micro-organisms.
2. Sterilization: Also suitable for extended fermentation as it inactivates almost all micro-organisms. May cause chemical changes in molecules due to heat (e.g., caramelization).
3. UHT (Ultra-High Temperature Processing): The standard method for preserving milk, offering excellent contamination control with minimal heat impact on the product.

Filtration: common practice, but less effective in combination with complex side-streams due to the presence of particles in such streams that can clog filters.

Chemical: used for equipment, like vessels, instruments and piping. Peracetic acid, hydrogen peroxide or isopropyl alcohol are examples of agents used for chemical sterilization of equipment. Each chemical has specific safety and practicality considerations.

Relevant Questions

- Does the side-stream require pre-treatment?
- Has the pre-treatment process been fully tested in combination with the rest of the process?
- What are the costs for sugar release, if this is a required step?
- Does the substrate contain components that negatively affect KPI's?

5. Fermentation process

Fermentation is a transformative process that converts a feedstock into valuable products, such as microbial biomass or specific proteins, using precision fermentation technology. Key performance metrics for **biomass** & **precision** fermentation include titer (product concentration), rate (production speed), and yield (efficiency of conversion). Depending on the specific fermentation type, additional factors may significantly impact the process.

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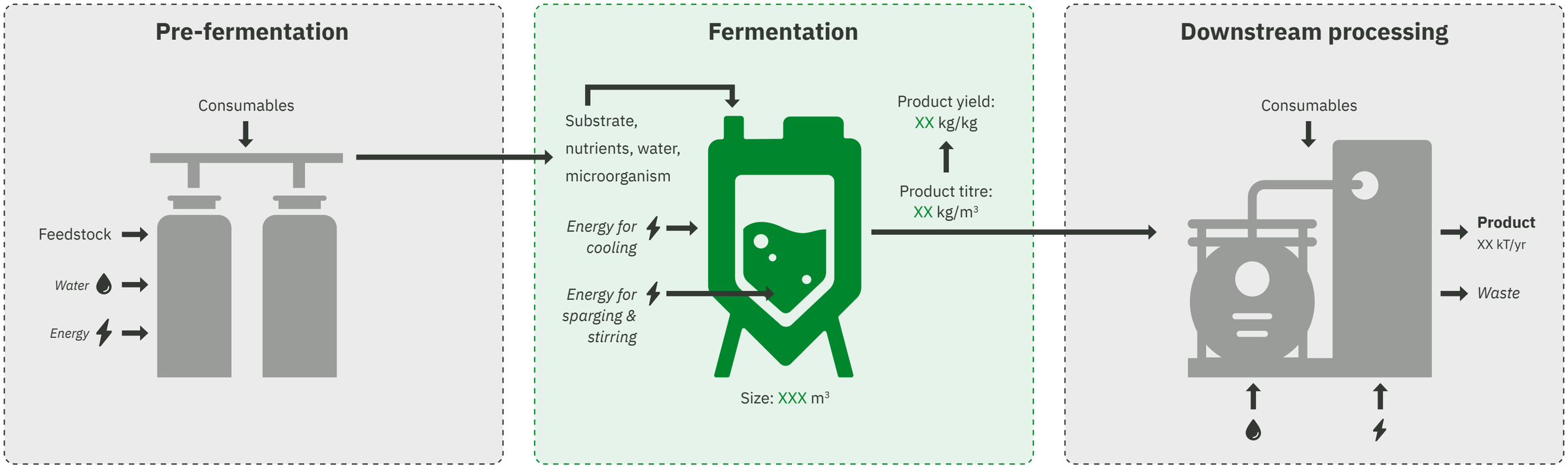
5. Fermentation process



Fermentation process configurations

Fermentation processes can generally be divided into three different configurations, or ‘modes’. With these modes, there is a trade-off between control over process performance on the one hand, and process complexity on the other. Microbial performance can be significantly impacted by this choice for process configuration, and performance in the one mode often does not translate directly to another.

Fermentation modes

Batch: All substrate(s) and other nutrients that are required throughout the process are present from the start; operated without adding or extracting any material to- and from the bioreactor (with the exception of e.g. acid/base addition for pH control).

- limited control.
- especially suitable when there is limited substrate- or product toxicity for the microorganism and product yields are high even during fast microbial growth.

Fed-batch: additional substrates and nutrients are fed into the bioreactor during operation (no outflow).

- control over microbial growth and product formation.
- requires a more advanced set-up of material and equipment.
- as there is no out-flow, the volume of the broth inside the reactor increases over time.
- typically lead to high product titers and are often used in precision fermentation or for aerobic production of microbial biomass.

Continuous fermentation: a feed of substrate(s) and nutrients into the vessel combined with continuous removal of produced biomass, product and residual nutrients.

- positive impact on microbial performance due to steady environment within bioreactor.
- can lead to a higher total productivity because of less down-time for cleaning and restarting.
- streams tend to be more diluted due to the continuous flow in- and out of the reactor.
  - advantageous in case of product toxicity.
  - may complicate product recovery in DSP.
- high risk of contamination and strain degeneration due to longer runtimes.

Relevant Questions

(Fed)-Batch fermentation

- Has the Fermentation process been thoroughly evaluated for the same mode as is intended for large scale production?
- What motivated the choice for a specific fermentation mode?
- How is the seed-train setup and has strain stability and contamination control been validated over the seed train?
- How is the end-of-batch time-point determined?

Continuous fermentation

- How is the risk of contamination mitigated and has this been tested at scale?
- Has the stability of the microbial strain and its performance been evaluated for long runtimes?
- Is the foreseen DSP suitable for continuous operation?

Aerobic versus anaerobic

Microorganisms may require oxygen to grow and produce products (aerobic), and some will not (anaerobic). Growth and product formation can differ significantly between aerobic and anaerobic processes. The choice for an aerobic or anaerobic process largely depends on the microorganism, and it has consequences for the yields that can be expected and for equipment requirements.

Oxygen and aeration or anaerobic processes

Aerobic processes: Biomass- and precision Fermentation processes are generally performed under conditions of high oxygen, because it is required for a high product yield on the feedstock. This requires complex aeration installations to ensure sufficient oxygen supply.

- oxygen availability should be carefully monitored because suboptimal oxygen supply can have a drastic negative impact on process performance.
- advanced cooling installations are required, because more energy is released during respiration (=using oxygen).

Anaerobic processes: For general fermentation mostly, anaerobic process are used. This minimizes oxidation reactions and thereby off-flavor formation.

- Such processes generally require less advanced installations for aeration and cooling.

Relevant Questions

? Does this concern an aerobic or anaerobic process?

Aerobic

? How sensitive is microbial performance to changes in oxygen availability?

? How will oxygen availability be monitored?

? What type of installation for aeration will be used?

Anaerobic

? How sensitive is the micro-organism used to oxygen and does this pose challenges at industrial scale?

Process monitoring & control

Introduction to fermentation control

To ensure that optimal conditions for microbial activity are maintained within a bioreactor during fermentation, parameters such as temperature, pH and oxygen availability are monitored and adjusted while the process is running.

In more advanced set-ups, changes in key performance indicators (KPI's) can be used as trigger for adjustments in process control (e.g. alter feed-profiles, aeration regimes, etc.).

It is important that the relevant KPI's for a specific process can be monitored with sufficient accuracy during operation to allow process control.

Monitoring tools and technology

Monitoring technologies can differ significantly in their TRL. A process can be monitored in time in different ways:

- Offline / at line: samples are extracted from reactor and analyzed elsewhere.
  - Well-established for:
    - Tracking microbial growth (biomass density);
    - Off-gas analysis;
    - Measuring organic compounds (HPLC or GC);
    - Analyzing protein production in precision fermentation (electrophoresis, chromatography, mass spectrometry).
- Online / inline: measurements are done directly in the process or in a closed loop, in real time.
  - Well-established for: pH, temperature, oxygen availability.

Emerging optical spectroscopy methods:
Techniques like near-infrared (NIR) or Raman-spectroscopy can monitor many different molecules within the reactor simultaneously and in real time. However, due to high equipment costs and the requirement for very specialized knowledge for data processing and interpretation they are not yet commonly applied for industrial scale fermentations (although there are some examples of using NIR).

Relevant Questions

- Are key performance indicators clear and measurable during the process?
- What is the TRL of the intended monitoring technologies?
- In case of advanced tools, is the required expertise available?
- Is process automation based on monitoring technology included in industrial design, and has this been evaluated at an appropriate scale?

Strain selection

Strain selection in general fermentation

Features that guide selection of microbial strains in this type of fermentation:

- Food-grade
- Capable of growing on the plant-based feedstock
- Introduces a favorable/desired flavor profile, texture or nutritional quality

Strain selection in biomass fermentation

Selection for strains used for biomass (or: microbial cell mass) production is based on:

- Food-grade;
- Capacity to grow to high cell densities (>100 gram dryweight/L);
- Favorable nutritional properties and/or flavor/texture of its own;
- Preferable: can already grow on relatively cheap substrates such as agricultural waste streams.

Strains with improved or novel capabilities are commonly selected based on high-throughput screening approaches using food-grade strain libraries.

- Development of high-throughput small-scale culturing methodologies should not be underestimated.
- The throughput of analyses that determine interesting capabilities should match the culturing throughput (if you perform >200 small-scale fermentations a week, you should also be able to measure >200 fermentations per week).

Relevant Questions

- Is the intended strain food-grade?
- What is the intended substrate?
- Does the strain fast enough to meet required annual production within the limits of the aimed bioreactor volume?
- Is it known whether the strain is able to produce any mycotoxins or endotoxins?

Microbial performance in biomass fermentation

For biomass fermentation the titer, rate and yield are the most important determinants for the overall process costs. In general optimization of 1 of these parameters may lead to a decrease in of the other parameters.

Titer: typically ranges between 100-200 g/L after process optimization. A high titer is crucial for efficient use of bioreactor volume.

Rate: The growth rate tends to range from 0.1 – 0.2 h-1 for fungi and 0.3 – 0.6 h-1 for yeast species. Bacterial growth rates can vary from 0.2 – 2.0 h-1.

Yield: typical values for (single cell) protein yield on (pure sugar) substrate range between 0.3-0.6 [g protein] / [g sugar]. The majority of the substrate will be converted to microbial biomass and CO2.

Titer, rate and yield can be improved through:

- Optimizing process configuration: fed-batch or continuous processes are often applied, to maximize both yield and growth rate by tuning the feed-rate.
- Medium optimization: optimal ratios between different types of nutrients may also steer the micro-organism towards higher yields and rates.

Relevant Questions

- Does the current information on titer, rate and yield show challenges to an economically viable process ? At what scale and under industrially-relevant conditions has the available info been acquired?
- What are the expectations for maximum values for titer, rate or yield (theoretical or otherwise)?
- Is there a clear idea of how upscaling effects impact these key performance indicators?
- Has a (preliminary) TEA already been performed based on accurately measured values for titer, rate and yield?

Strain selection – Precision fermentation

‘Chassis’ microorganism for protein expression

In precision fermentation, microorganisms are mostly used as a very small scale ‘factory’ for protein production. Therefore, they are often also referred to as ‘chassis’ microorganisms or ‘microbial host’.

Features that guide selection of a suitable microbial host include:

- Ability to grow well on relatively cheap media;
- Ability to produce large amounts of protein;
- Ease of genetic modification;
- Ideally, produced proteins do not remain within the cell but can be secreted (this can depend on the host, but also on the protein itself);
- Ability to introduce post-translational modifications (if required – see slide 27).

Commonly used microbial hosts for different types of microorganisms are listed below:

Yeasts
Saccharomyces cerevisiae
Kluyveromyces lactis
Yarrowia lipolytica
Pichia pastoris (Koma-gataella phaffii)

Fungi
Aspergillus spp.
Trichoderma reesei

Bacteria
Escherichia coli
Bacillus spp.

Relevant Questions

- Has a host been selected for production of the protein of interest?
- What hosts have been evaluated?
- On what basis was the selected host chosen?
- Is the intended host GRAS and/or QPS?
- Is the product secreted into the medium?

Strain construction - Precision fermentation

Basic strain construction

A selected microbial host needs to be genetically modified to be able to produce the protein of interest, which it does not produce naturally. The gene (DNA) that encodes for that specific protein needs to be introduced. This involves the following steps:

- Vector construction: this is a piece of DNA that contains the code for the protein, and all elements required for stable production of that protein. This is usually a modular design, in which each element (allowing for e.g. selection, overproduction, secretion) can be optimized.
- Introduction of the vector DNA into the microbial host ('transformation'). The efficiency can vary significantly depending on the host, which can impact future optimization endeavors.
- Vector maintenance: the DNA needs to be kept inside of the microbial host cells.
  - Some vectors remain as loose elements in the cell and require continuous selection, for example through the use of antibiotics.
    - (risk of losing this DNA and use of antibiotics may not meet regulatory requirements!).
  - Others are, after introduction and initial selection, stably integrated into the genome and remain there afterwards.

Relevant Questions

- Are genetic tools for efficient engineering of the selected host available?
- Is the gene stably integrated into the host genome?
- Is strain engineering without antibiotic selection possible, or can such marker genes be efficiently removed in due time (in view of possible regulatory requirements in this respect)?
- Does the host/vector system allow efficient secretion of the protein?
- What methods are used for verification of protein production?

Source	DNA	Genetic Modification	Microbial Host	Protein
• Genetic sequences expressing animal or plant-based proteins • Data mining of large DNA-sequence databases	• Gene expression constructs are usually synthesized in-vitro • Codon optimization is usually tailored to the microbial host	• Integration of gene construct using molecular toolbox (e.g. CRISPR-Cas) • Multiple copies often necessary	• Microbial host will produce the protein inside the cell • Secretion of the protein into the extracellular medium highly preferred	• Depending on the protein/host a purity of >95% can be achieved • In some cases, DSP can be kept minimal when using GRAS hosts

Strain engineering and development

Post translational modifications

For some proteins, simply producing the protein itself is not enough to achieve the desired functionality. These require additional chemical modifications after production to gain full functionality. These modifications, known as post-translational modifications (PTMs), can add value by enhancing protein activity, stability or general functionality. Examples of PTMs are:

- Glycosylation (the attachment of sugar-like molecules).
- Phosphorylation (attachment of phosphate groups).
- Proteolytic maturation (partial degradation of the protein).

Advanced strain engineering

To produce a specific protein in sufficient quantities and in the right form, the microbial host itself may need to be further modified.

- Some hosts are naturally able to introduce PTMs, but the patterns often deviate from those in the original animal protein. Editing of the DNA of the host itself may be required to change PTMs.
- Achieving efficient secretion of some proteins may require the introduction or deletion of specific genes.

When it is not or insufficiently known which genes need to be added, removed or edited to optimize strain performance, random mutagenesis may be applied in combination with screening.

- Mutagens (chemicals, UV) are used to introduce random mutations in the genome of the microbial host. Most of these methods are not considered GMO.
- Screening is then used to identify mutant strains with improved performance. A good read-out for protein production (or functionality, or specific PTM pattern) is then required.

All these aspects of adjusting the microbial host to improve performance are routinely evaluated in so-called Design-Build-Test-Learn (DBTL) cycles.

Relevant Questions

- Is there a clear vision on how to approach strain improvement activities?
- Is there a good method for quantification of the protein?
- Are complex PTMs required?
- Is further engineering of the microbial host required, to obtain specific PTMs or otherwise?
- Is there a good method to validate protein functionality?
- Is (random) screening involved in strain optimization?

5. Fermentation process

Microbial performance

Especially for precision fermentation, the concepts of titer, rate and yield and the trade-offs between those come into play.

Titer: typically ranges between 1-20 g/L after optimization (very much dependent on the type of protein) but may be much lower in early stages of development. A high titer is crucial for efficient DSP, and therefore optimization of titer is essential.

Rate: variable per type of microbial host and protein that is produced.

Yield: typical values for protein yield on the sugar feedstock ranges between 0.005-0.05 [g protein] / [g sugar]. The majority of the substrate will be converted to microbial biomass (growth, new cells) and CO2.

Titer, rate and yield can be improved through:

- Engineering of the microbial host (the flow of substrate within the cells is redirected towards protein production, rather than growth).
- Optimizing process configuration: fed-batch processes (see slide 20) are often applied, because growth can be actively reduced and protein production boosted through clever use of the feed-rate.
- Medium optimization: optimal ratios between different types of nutrients may also steer the micro-organism towards more protein production rather than growth.

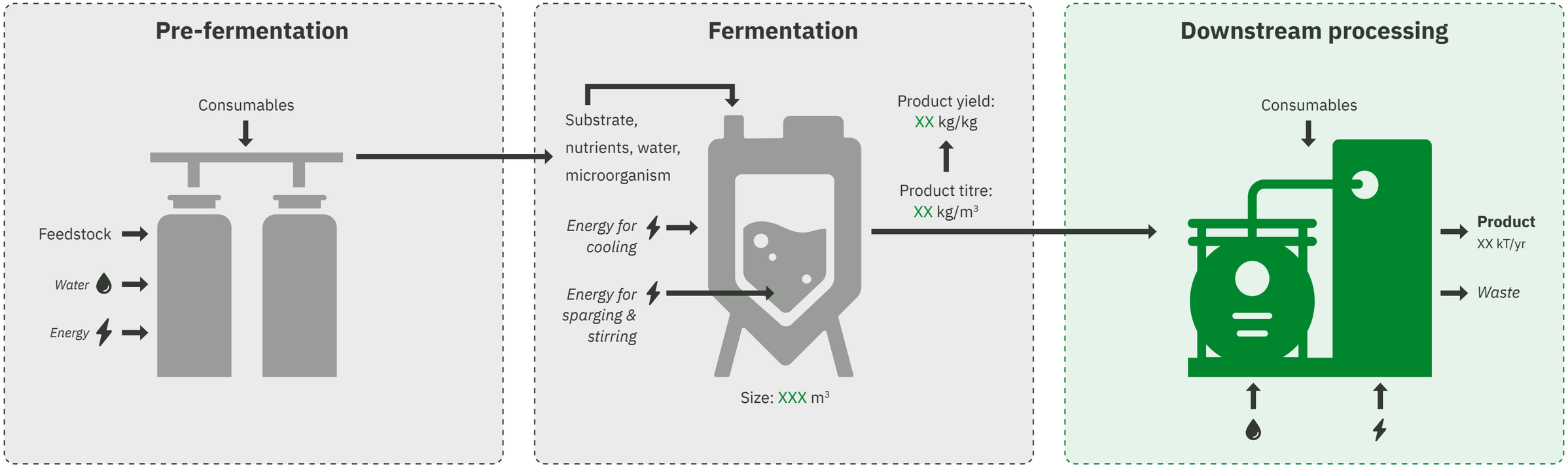
Relevant Questions

- Are clear (current) values for titer, rate and yield available?
- Does the current information on titer, rate and yield show challenges to an economically viable process ?
- At what scale and under which (industrially-relevant) conditions has the available information been acquired?
- What are the expectations for maximum values for titer, rate or yield (theoretical or otherwise room for improvement)?
- Is there a clear idea of how upscaling impacts these key performance indicators?
- Has a (preliminary) TEA already been performed based on accurately measured values for titer, rate and yield?

6. Down Stream Processing (DSP)

Depending on the required purity of the product, downstream-processing (DSP) can represent a considerable fraction of the total production costs. It is therefore important that the DSP is efficient (minimal loss of product, high purity, low operating costs). DSP as defined here involves biomass harvesting or removal, and product purification, concentration, and stabilization (i.e., product formulation is not considered here, although that is potentially an important cost factor also).

6. Down Stream Processing (DSP)



Fermentation product quality

Altering flavor, texture and other aspects of food products using fermentation is widely used. Sensory perception by humans is extremely sensitive and ensuring reproducibility of sensorial and nutritional aspects of fermentation outcomes can be challenging. Maintaining product similarity over time therefore requires careful process control and product analysis.

Analysis of flavor, texture and nutritional value

Flavor and **texture** are very complex aspects of a food product, and many different types of analyses can be involved to get a clear picture.

- Laboratory (physical and chemical) analyses can be used to monitor whether attributes remain within pre-determined bounds, but not (yet) to make accurate predictions about sensory perception.
- For development of new flavor and texture, testing by trained panels will often be involved, as well as consumer studies.

Fermentation may change the **nutritional** value of a food ingredient both in a positive and negative way.

Examples of positive effects are:

- lowering sugar concentrations;
- increasing acid and (potentially) vitamin concentrations;
- improved digestibility due to (partial) breakdown of complex nutrients by microorganisms.

Factors impacting robustness of product quality

In order to operate a reliable and stable process at large scale, inconsistent flavor and texture outcomes should be avoided. Factors that can impact product quality include:

- **Fermentation conditions** (e.g. temperature, pH, oxygen availability) impact the performance of the microbial culture and hence the type of modifications that are introduced in the feedstock.
- **Feedstock composition** can be very determining for the outcome of the fermentation, since ultimately, microorganisms convert precursor molecules originating from the feedstock into components relevant for flavor, texture, nutrition etc.

Relevant Questions

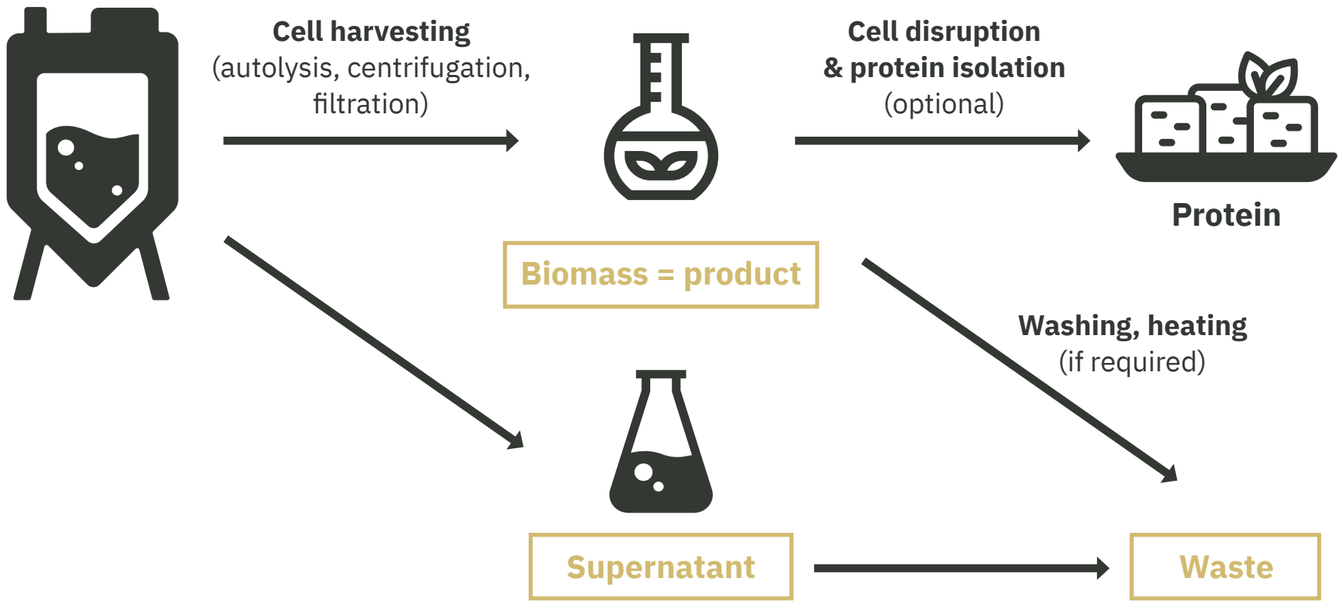
- Are there clear and measurable quality parameters for flavor, texture and/or nutritional value?
- Has a sensitivity analysis been conducted to assess how batch-to-batch variations in feedstock impact key product attributes (e.g., flavor, texture, nutrition)?
- Has a sensitivity analysis been performed on how Fermentation process conditions impact key product indicators?
- Has research been done on how fermentation influences the nutritional profile of the final product?
- How is microbial activity stopped at the end of fermentation?
- Are any other processing steps required to stabilize the product or optimize important quality parameters?

Biomass fermentation

Importance of downstream-processing

In biomass fermentation, biomass is the product and it needs to be separated from other components after fermentation:

- Cell harvesting: main DSP requirement (centrifugation, filtration).
- Washing and heating: may be necessary to remove remaining medium components.
- Optional: cell disruption and protein isolation (in some cases, only the protein is desired rather than the entire microbial cell).
- The residual water after removing the biomass are in general disposed by a waste-water treatment plant. These tend to be large volumes and therefore incur costs.



Relevant Questions

- Is total biomass the product or total protein?
- If total protein, what methods are used for isolating the total protein fraction?
- Can waste streams be valorized?
- What is the suggested DSP to USP cost ratio at scale?

Quality of produced Biomass

Biomass production typically relies on yeast or fungal fermentation, though some start-ups also explore bacterial biomass. These micro-organisms exhibit variations in texture, sensory properties, and associated risks.

Bacteria

In the 1970s, a large-scale fermentation plant was established to cultivate bacteria on methanol as a source of single-cell protein for animal feed. Today, several companies are exploring the potential of bacterial biomass for food applications. Bacteria are advantageous due to their high growth rates; however, a drawback is that many species produce endotoxins.

Yeast

Yeast biomass production is a well-established industrial process, particularly for its use as a flavoring agent in various food products. After fermentation cells are disrupted (autolysis)to allow proteins to become available and separate out insoluble by separation and filtration. Depending on the flavor required the process needs to be well defined and specified as such steps may cause or aim to remove specific off-flavors generated.

Fungal Biomass

Fungal biomass, especially from filamentous fungi, is valued for its fibrous texture, which can replicate the chewiness of meat and is thus popular in meat alternatives. This unique texture, however, can be inconsistent due to the continuous nature of fungal fermentation, leading to variability between and within batches. Quality control measures are essential to ensure the final product meets texture and mouthfeel standards. Fungal species can produce mycotoxins under certain conditions, posing a health risk.

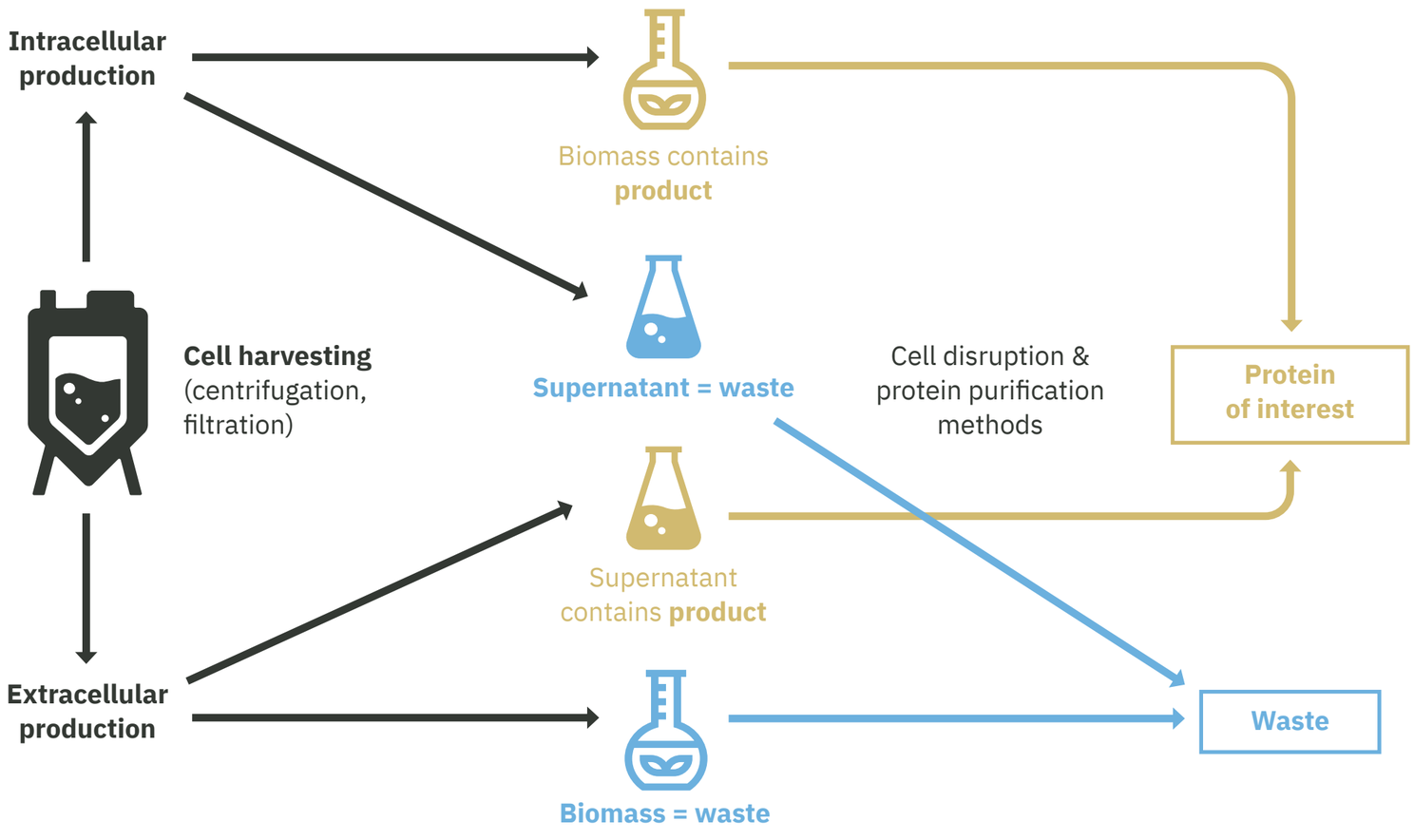
Relevant Questions

- Is there a clear view of which functional parameters are required for value creation of the product
- Which parameters influence the functionality (flavor, texture, etc) of the produced biomass
- Has the bacterial or fungal biomass been tested for toxin production in (a lab mimic of) the industrial setting?

Precision fermentation

Importance of downstream-processing

In precision fermentation the specific protein of interest represents only a small fraction of the whole material after fermentation. Elaborate DSP is typically needed to isolate and purify the protein and to remove potential contaminants that originate from the microbial host.



Relevant Questions

- Is there full clarity on the protein purification process?
- Is the protein of interest produced intra- or extracelullarly? Why was this choice made?
- What purity level is needed?
- Which protein purification technologies are envisioned?
- Is the chosen downstream processing methodology compatible with product stability?
- How is complete removal of the GMO cells from the product ensured?
- At what scale has DSP been demonstrated?
- What is the recovery of the DSP process (the fraction retrieved)?

Quality control in DSP

Ensuring consistency in color, texture, and functionality

Batch-to-batch product quality differences, and fluctuations during continuous processes, should be avoided. Means of including monitoring quality include:

- Contamination should be checked to ensure food safety. This is generally done using colony counts to check for microbial contamination;
- Absence of GMO-derived DNA, which is a common regulatory requirement, can be verified by quantitative PCR;
- Protein quality and average folding can be checked with various technologies, that each do not provide a whole picture, such as gel-based analysis for protein size, differential scanning calorimetry, circular dichroism spectroscopy, size-exclusion chromatography and many more. All these technologies require specific experts;
- Product-specific functionality assays such as shear and rheology testing, waterholding capacity, viscosity measurements or texture profile analysis (mimics chewing).

Relevant Questions

- What aspects of product quality would need to be monitored?
- How is this achieved in practice, and what are the costs?
- Is their sufficient technological expertise on the team to test product functionality?
- What purity level is needed?
- At what scale has DSP been demonstrated?
- What is the recovery of the DSP process (the fraction retrieved)?
- Can certain DSP waste streams be valorized? If not, what are the costs of waste disposal?
- Is absence of host-derived DNA required in the target market countries? How is this achieved?

Product stability (protein)

Stability challenges during storage and transport

Proteins often need to be stabilized during storage and transport. Proteins may suffer from:

- Aggregation/unfolding (either spontaneous or caused by heating);
- Microbial growth (commonly also causing proteolytic degradation);
- Proteolytic degradation caused by host-derived proteases that were not completely removed.

To ensure proteins remain stable throughout their journey to market, it's essential to consider these factors and test for stability during logistics.

Common means of stabilization

- Freezing: Helps preserve structure and function.
- Drying: Techniques like spray drying or lyophilization.
- Stabilizing Agents: Added to enhance stability.
- pH Adjustment: A cost-effective option when applicable.
- Enhanced Purification: Removing impurities for improved stability.

Relevant Questions

- What testing protocols are in place to validate protein stability?
- What is the projected shelf life of the protein under typical storage and transport conditions?
- In what ways does the company mitigate risks associated with microbial contamination during transport
- How does the company ensure quality control to monitor aggregation, unfolding, or degradation over time?
- Which stabilization methods are currently used, and why were they chosen over other options?
- Which stabilizing agents are used and to how do these impact costs, environmental aspects or labelling of the product?

Waste handling

Precision fermentation generates significant waste streams of GMO biomass. Typically, over 90% of the supplied carbon is converted to biomass, with less than 10% used for the target protein.

Applications

Animal Feed:

- High protein content offers potential for animal feed.
- Strict regional regulations apply to GMO biomass usage.

Valorization:

- Emerging uses include biofuels, bioplastics, and soil amendments.
- These applications remain in early developmental stages.

Disposal Methods:

- Options include composting, anaerobic digestion, or incineration.
- Balancing sustainability, compliance, and cost is critical.

Key Considerations

- Evaluate sustainability vs. costs of waste handling.
- Address regional regulatory requirements for waste use.

Relevant Questions

- Is there a clear strategy for handling the GMO biomass waste generated in precision fermentation?
- How does the company ensure regulatory compliance for waste biomass?
- How does the company handle regulatory differences across regions?
- Can the biomass waste be valorized?
- How does the cost of waste handling impact the company's overall profitability?

7. Scalability and industrial considerations

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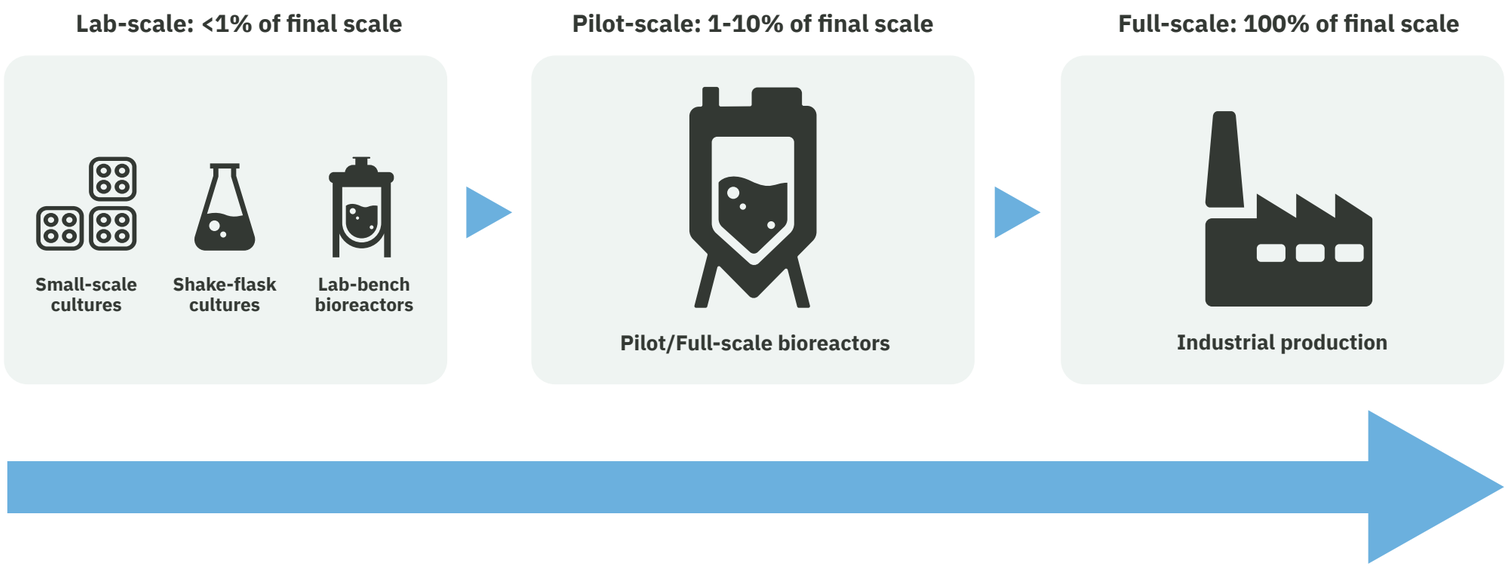
Scaling & scalability challenges

Oxygen transfer, mixing, and heat distribution at scale

Oxygen transfer, mixing, and heat distribution are critical at industrial scales for biomass and precision fermentation. Larger bioreactors often experience oxygen depletion at the top, reducing yield. Understanding and mitigating this is vital for optimal performance.

Considerations for continuous/fed-batch processes:

- Continuous feeding can create localized concentration variations, impacting microbial efficiency.
- Validating sensitivity to these fluctuations ensures consistent output and efficiency.



Relevant Questions

- Has microbial performance been tested at relevant scales?
- In which reactor type has the process been tested and how does this compare to the industrial scale process ?
- Has the sensitivity of KPI's for potentially fluctuating parameters (oxygen concentration, feedstock concentration, temperature) been assessed?

Regulatory compliance and food safety

Various types of microorganisms are used in fermentation, and regulatory requirements for their use vary by region. In some areas, specific microbial strains fall under the Nagoya Protocol, meaning they may be subject to intellectual property (IP) rights associated with their country of origin. Understanding these regulatory nuances is crucial for investors to assess market entry risks.

Regulatory consideration

- Key regulatory frameworks include GRAS (Generally Recognized as Safe) in the U.S. and QPS (Qualified Presumption of Safety) in the EU, with specific rules governing both novel food applications and genetically modified organisms (GMOs).
- Nagoya Protocol Compliance: Countries that adhere to the Nagoya Protocol impose restrictions on certain microbial strains, potentially linking them to the IP of the source country. Verifying compliance with this protocol is essential when using specific strains.
- Regional Approval for General Fermentation processes: The EU may allow general Fermentation processes if a similar process was available before a specified historical cutoff, while the U.S. applies different standards for market approval.
- Regional Approval for Biomass Fermentation processes: In the EU, specific yeast (e.g., Saccharomyces cerevisiae) and fungal species (e.g., Fusarium venenatum) are approved for biomass fermentation, with similar approvals in other regions like the U.S., China, and parts of Asia. However, local regulations may vary. Other species may not be approved depending on local regulations.
- Precision Fermentation and Novel Foods: Precision fermentation products are generally classified as Novel Foods, requiring regulatory approval in the EU, U.S., and other markets before commercialization.

Relevant Questions

- How does the company ensure compliance with GRAS (U.S.) or QPS (EU) status for their products?
- Have GRAS or Novel Food approvals been obtained? If not, what is the strategy and timeline?
- Does the company work with GMO strains, and if so, how does it navigate differing GMO regulations across regions?
- Are any of the microbial strains or genetic resources used subject to the Nagoya Protocol, and if so, how does the company ensure compliance?
- How does the company handle variations in regulatory standards between the U.S., EU, and other target markets?

R&D infrastructure

Shared research and pilot facilities

Investing in pilot facilities entails significant capital expenditures (CAPEX). Partnering with specialized service providers can mitigate these costs by offering access to advanced facilities and expertise. Notable organizations include:

- Wageningen Food & Biobased Research (WFBR): Provides a range of laboratory and pilot facilities, with capacities up to 100 liters. WFBR specializes in reproducible scaling of processes using advanced technologies.
- NIZO: Offers contract research organization (CRO) services focused on process optimization and scaling, with facilities ranging from 45 to 4,000 liters. NIZO's in-house capabilities facilitate a high success rate and reduced development time for new processes and products.
- BBEPP (Bio Base Europe Pilot Plant): Provides comprehensive CRO and contract manufacturing organization (CMO) services, supporting scale-up from pilot to demonstration levels, with capacities between 35 and 75,000 liters.
- Plant One: Offers a variety of pilot-scale equipment available for rent, enabling process development and testing without substantial capital investment.

Industrial location

The location of the final industrial process is likely to be key for running a profitable business. The following considerations are of importance:

- Vincinity production location to the end-product manufacturers (e.g. co-locate a precision fermented dairy protein should be co-located with dairy industry).
- Vincinity to the side-stream producer as transports costs of side-streams are generally very high.
- Ensure sufficient grid power availability at the location as these can be energy intensive processes.

Relevant Questions

Shared Facilities

- Are shared research and pilot facilities being leveraged to reduce CAPEX?
- What scale of pilot testing has been performed, and with which providers?
- How have these facilities contributed to scaling efficiency?

Industrial Location

- Is the selected location optimized for proximity to manufacturers and feedstock suppliers?
- What is the strategy for minimizing transport costs of side-streams?
- Is there adequate infrastructure (e.g., grid power) to support large-scale operations?

8. Final considerations

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Intellectual property and competitive position

Protecting the process or product is critical to secure a return on investment and ensure long-term competitiveness. Strong IP protection, product novelty, and technical uniqueness strengthen an organization's market position.

IP position

- A strong intellectual property position is essential for safeguarding the process or product. Companies should secure patents or other forms of protection to maintain exclusivity and competitive advantage.
- Strategies to enhance IP, such as licensing agreements, add value to the company's position.
- Complex and unique processes reduce the risk of replication by competitors, even in the absence of direct IP protection.

Product novelty

- A distinctive product stands out in the market by offering unique features or addressing unmet needs.
- Differentiating factors, such as enhanced functionality, cost efficiency, or sustainability, play a critical role in market positioning.
- Novel products add value by appealing to niche markets or creating new market opportunities.

Technical uniqueness

- Proprietary techniques or innovative production methods create a competitive edge by improving efficiency, scalability, or cost-effectiveness.
- Unique technical approaches may act as barriers to entry for competitors.

Relevant Questions

Shared Facilities

- Are shared research and pilot facilities being leveraged to reduce CAPEX?
- What scale of pilot testing has been performed, and with which providers?
- How have these facilities contributed to scaling efficiency?

Industrial Location

- Is the selected location optimized for proximity to manufacturers and feedstock suppliers?
- What is the strategy for minimizing transport costs of side-streams?
- Is there adequate infrastructure (e.g., grid power) to support large-scale operations?

8. Final considerations

Why invest in fermentation now?

The fermentation market is rapidly growing as consumers demand sustainable and innovative food solutions. Driven by the rise of alternative proteins and advancements in scalable, cost-effective biotechnology, fermentation addresses global challenges like food security and climate change. With the market projected to reach $1.25 trillion by 2034 and an estimated CAGR of 8.1% over the next decade it offers significant investment potential across diverse sectors.

Growth Potential

- Scalability: Advancements in fermentation technology are enabling ever more cost-effective, large-scale production.
- Diverse Applications: market opportunities in food applications keep on expanding as knowledge, especially on taste, in the plant-based food space keeps increasing.
- Investment Returns: Complex high-tech applications and increasing market adoption may be expected to allow for strong long-term profitability.

Sustainability Impact

- Environmental Benefits: Low resource usage, reduced greenhouse gas emissions, and minimal waste.
- Circular Economy: Fermentation allows for upcycling of by-products and side-streams for economic and ecological gains.
- Future-Ready: Addresses global challenges of food security, climate change, and resource scarcity.

Annex

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Annex I: Key Terminology in Fermentation

- *Host Organisms:* Microbes such as bacteria, yeast, or fungi engineered to produce desired compounds.
- *Genetic Engineering:* The manipulation of an organism's DNA to introduce new traits or capabilities.
- *Synthetic Biology:* An interdisciplinary field combining biology and engineering to design and construct new biological parts and systems.
- *Recombinant DNA Technology:* Techniques used to combine DNA from different sources into a single molecule to produce new genetic combinations.
- *Expression System*: The combination of genetic constructs and host organism used to produce a target protein.
- *Fermentation process:* Cultivating microorganisms under controlled conditions.
- *Bioreactor:* A vessel or system that provides a controlled environment for fermentation, optimizing factors like temperature, pH, and oxygen levels.
- *Upstream Processing:* Steps involved in preparing and running the fermentation, including media preparation and inoculation.
- *Downstream Processing:* The purification and extraction of the desired product after fermentation.
- *Metabolic Engineering:* Modifying metabolic pathways within an organism to increase production yield of a target compound.
- *CRISPR-Cas:* A genome-editing tool used to make precise changes in the DNA of organisms
- *Cell Factory:* Engineered microorganisms designed to efficiently produce a specific product.
- *Scale-Up:* The process of increasing production volume from laboratory scale to industrial scale while maintaining efficiency and product quality.
- *Product Titer:* The concentration of the desired product in the fermentation broth.
- *Specific biomass yield:* The amount of biomass produced per unit of substrate consumed.
- *Specific product yield:* The amount of product produced per unit of substrate consumed.
- *Substrate:* The raw materials (often sugars) used by microorganisms during fermentation.
- *Batch Fermentation:* A closed-system fermentation where all ingredients are added at the beginning, and no additional inputs (other than control elements) are added during the process.
- *Continuous Fermentation:* An open-system fermentation where substrates are continuously added, and products are continuously removed.
- *Fed-batch Fermentation:* A partly open system fermentation where nutrients are being fed after an initial batch phase to enhance growth and product yield.
- *GMO (Genetically Modified Organism):* An organism whose genetic material has been altered using genetic engineering techniques.
- *DBTL cycle:* design-build-test-learn approach in synthetic biology, with the aim of improving strain properties through repeated genetic interventions
- *Adaptive laboratory evolution (ALE):* methodology for adapting (evolving) strains to selective environmental conditions through repeated transfer of cells or continuous culture
- *Polysaccharides:* Complex carbohydrates composed of long chains of sugar molecules. In fermentation, they serve as a substrate or structural component, influencing microbial growth and product synthesis.
- *Medium:* The nutrient solution used to support the growth of microorganisms during fermentation. It typically contains carbon sources, nitrogen, minerals, and other growth factors.
- *Feedstock:* The raw material or substrate used as the primary energy and carbon source in Fermentation processes. Examples include sugars, starch, agricultural waste, or industrial by-products.

Annex II: References for further reading

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Slide 51 (Investor Considerations): Future Market Insights (2024). Fermented Foods and Beverages Market: Industry Analysis and Opportunity Assessment 2024-2034. Retrieved from https://www.futuremarketinsights.com/reports/fermented-foods-and-beverages-market.

General further reading (book applicable to most slides)

Waites, M. J., Morgan, N. L., Rockey, J. S., & Higton, G. (2009). Industrial microbiology: an introduction. John Wiley & Sons.

https://gfi.org/fermentation/