FACULTY OF
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# THE FLAVOUR OF MICROBIAL PROTEIN

How protein content and flavour are affected by different micro-organisms and carbon sources in batch growth

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# ABBREVIATIONS

| | |
|---|---|
| AA | Acetic acid |
| AAA | Aromatic amino acids |
| COD | Chemical oxygen demand |
| Exp | Exponential growth phase |
| ExpL | Late exponential growth phase |
| FA | Formic acid |
| FAO | Food and Agriculture Organization of the United Nations |
| FID | Flame ionisation detector |
| HOB | Hydrogen-oxidising bacteria |
| IC | Ion chromatography |
| MP | Microbial protein |
| OD | Optical density |
| $P_P$ | Protein productivity |
| $P_X$ | Biomass productivity |
| PC | Principal components |
| PCA | Principal components analysis |
| RI | Retention index |
| SAA | Sulphuric amino acids |
| SCP | Single cell protein |
| Stat | Stationary growth phase |
| TSS | Total suspended solids |
| VOC | Volatile organic compound |
| VSS | Volatile suspended solids |
| $Y_P$ | Protein yield |
| $Y_X$ | Biomass yield |

# ABSTRACT

As the world population increases, it becomes continuously more challenging to provide sufficient food and to do so sustainably. Especially high quality protein demands increase rapidly due to the wealth increase in developing countries and concomitant rise in consumption of animal-based protein, such as meat and dairy. However, these resource-intensive products induce a growing competition for the planets finite natural resources, which is ultimately not sustainable. To this end, the possibility to use protein of microbial origin has been explored because of its minimal environmental impact and high protein quality. Yet, minimal knowledge is at hand about the taste or smell of microbial protein. The scope of this research was thus to assess the microbial protein quantity and flavour, and how these might be affected by cultivation conditions.

In order to frame the performed research, a literature study was performed, discussing the applicability of microbial protein and the assessment of flavour.

In a first section, the influence of the micro-organism and supplied carbon substrate on the protein were assessed in batch growth. Overall, both of these parameters had a significant influence on the protein content and concentration. Moreover, the choice of the micro-organism had a more influencing impact on the protein levels than the change in carbon source.

Next, the protein content was evaluated throughout the different phases of the bacterial batch growth. It was hypothesised that the protein content would reach a maximum during the exponential phase, however this trend was not observed. Instead, the protein production rate reached its maximum during this phase and the protein content exhibited either a continuous increase or remained rather constant throughout the growth. This was also confirmed by the analysis of the protein productivity through time. The growth rate appeared to be more influential than the growth phase.

The third part of this thesis involved the use of an electronic nose (eNose) to assess the flavour of each sample. To our knowledge, this was the first time such a technique was applied for microbial protein and it was demonstrated that the eNose could effectively discriminate between samples. The choice of organism seemed to be more influential on the aroma than the choice of carbon source.

Lastly, an attempt was made to identify the volatile compounds present in the microbial protein aroma through the selection of 20 components that accounted for the highest variance between samples. As this identification was performed on the basis of Kovats retention indices calculated with experimental retention times, these components could

only be identified tentatively. It is thus not guaranteed that these are actually present in the samples' aroma profiles. These components could also be linked to their respective aroma descriptors.

The use of the eNose was a promising first attempt at unravelling the aroma of microbial protein and how it might be influenced by cultivation conditions.

# SAMENVATTING

Door de steeds groeiende wereldbevolking wordt het steeds uitdagender om voldoende voedsel te voorzien, maar ook om dit op een duurzame manier te produceren. Vooral de vraag naar proteïne bronnen van hoge kwaliteit stijgt sterk door de stijging in rijkdom in ontwikkelingslanden en de daarmee samenlopende toename in consumptie van dierlijke producten. Deze grondstof-intensieve producten brengen echter een toenemende competitie teweeg om de eindige natuurlijke grondstoffen van de Aarde. Het gebruik van microbiële eiwitten als alternatieve eiwitbron werd daartoe onderzocht omwille van zijn minimale impact op het milieu en hoogwaardige eiwitsamenstelling. Aan de andere kant is er slechts weinig geweten over de smaak of geur van dit product. Het doel van dit project was dan ook om deze smaak te bepalen alsook de eiwit kwantiteit en hoe deze beïnvloed kunnen worden door verscheidene groeiomstandigheden.

Het gedane onderzoek werd eerst gekaderd door een literatuurstudie, waarin de toepasbaarheid van microbieel proteïne werd besproken, alsook technieken voor het bepalen van smaak en geur.

Eerst werd de invloed van het gekozen micro-organisme en de koolstofbron op het proteïnegehalte in de biomassa en de totale proteïne hoeveelheid. Beiden bleken een significante impact te hebben op deze parameters, waarbij het gekozen micro-organisme het meest invloedrijk was.

In een volgend deel werd het proteïnegehalte ook geëvalueerd doorheen de verschillende fases van de bacteriële groei. Volgens de oorspronkelijke hypothese zou dit gehalte het hoogst zijn tijdens de exponentiële groeifase, hoewel dit niet werd geobserveerd. In plaats daarvan was de proteïne productie het hoogst in deze fase en bleek het proteïnegehalte ofwel continu te stijgen of bleef het enigszins constant. Dit werd ook bevestigd door de analyse van de proteïne productiviteit in functie van de tijd. De groeisnelheid bleek in dit geval meer impact te hebben dan de groeifase.

Het derde deel van deze thesis omvatte het gebruik van een elektronische neus om de smaak van elk staal te bepalen. Voor zover geweten, was dit de eerste keer dat een dergelijke techniek werd toegepast op microbieel proteïne. Er werd aangetoond dat de eNose afdoend onderscheid kon maken tussen de verschillende stalen. De keuze van micro-organisme bleek meer impact te hebben op het aromaprofiel dan de koolstofbron.

Als laatste werd getracht om de volatiele componenten te identificeren die in het microbieel proteïne aroma aanwezig waren op basis van een selectie van de 20 componenten die voor de meeste variatie in de aromaprofielen zorgden. Aangezien deze identificatie was

gebaseerd op Kovats retentie indices bepaald met experimentele retentietijden, konden deze componenten niet met zekerheid worden bepaald. Het is dus niet gegarandeerd dat de bepaalde componenten ook effectief aanwezig waren in het aroma. Deze volatielen werden nadien ook gelinkt aan hun respectieve aroma descriptoren.

Op deze manier bleek het gebruik van de eNose een veelbelovende eerste poging tot het ontrafelen van het aroma van microbieel proteïne en hoe dit beïnvloed wordt door groeiomstandigheden.

# I. INTRODUCTION

With the total world population estimated to reach 10 billion by 2050, the current food production rate will not be sufficient to cover the demand. To sustain this number of people, the amount of produced food calories will have to increase by roughly 70% between 2006 and 2050 (World Resources Institute, 2013). Food demands will not only increase due to population growth, but also due to the expansion of the global middle class in developing countries (World Resources Institute, 2016b). As the income of these families increases, so does their spending budget for groceries, resulting in a higher food consumption and thus demand (Springmann et al., 2018).

Another trend seen among new middle class households, is the adoption of western dietary habits with higher meat consumption (Springmann et al., 2018). The demand for animal-based protein, such as meat and dairy, has therefore known a quick rise. In some regions, meat consumption has even increased by 280% per capita from 1963 to 2013 (FAO, 2013b). The worldwide demand for products of animal origin continues to rise and is expected to be 80% higher in 2050 than in 2006 (World Resources Institute, 2016b).

These global changes in population size and diet composition do not only challenge the ability to produce sufficient amounts of food, but also to also do so in a sustainable manner. The growing competition for natural resources, as well as the changing climate, pose a real threat to the food production system. Arable land restrictions force the industry to improve production efficiencies: do more with less. Especially animal-derived products are more resource intensive due to the low conversion efficiency of plant-based feeds into animal matter (approximately 10%) (Godfray et al., 2010). The environmental impact of food production is further raised by greenhouse gas emissions, which are also higher when producing animal products compared to plant-derived products (World Resources Institute, 2016b).

It is clear that the current dietary habits and agricultural practices will not sustain an adequate food supply for the future generations. For this reason, the food industry is searching for alternative protein sources that have a lower environmental impact. These new, high protein ingredients could either be incorporated at a feed level or directly for human consumption. This has proven to be quite challenging, as the protein quality and consumer perception is of equal importance (Anupama et al., 2000).

To this end, the possibility to use protein of microbial origin has been explored because of its minimal environmental impact and high protein quality (Pikaar, Matassa, Bodirsky, et

al., 2018). Even though this research started during the 1960's, minimal knowledge is at hand about the taste or smell of microbial protein. In order to successfully incorporate this ingredient in food or feed, this information is of utmost importance. The scope of this research was thus to assess the microbial protein quantity and flavour, and how processing conditions might affect this.

# II. LITERATURE REVIEW

## 1. Microbial protein

### 1.1 History

Microbial protein (MP), also known as single cell protein (SCP), is protein originating from algae, fungi, yeasts or bacteria. Although consumption of MP might sound very futuristic, it is not a new concept. In fact, Imperial Chemical Industries launched a commercially available MP in the 1970's, the animal feed Pruteen™ (Braude et al., 1977). However, the Pruteen™ production was discontinued in the 80's due to high costs and competition from more cost-effective protein sources such as soybean (Kunasundari et al., 2013; Pikaar, Matassa, Bodirsky, et al., 2018). Eventually the overall attention for MP decreased and commercialisation was no longer pursued (Pikaar, Matassa, Bodirsky, et al., 2018).

Nevertheless, as the increasing world population and concomitant need for sustainable agriculture became more evident, the interest for MP has been revived in recent years. Advances in biotechnology and reactor technology have also reduced production costs significantly, enabling further commercialisation (Pikaar, Matassa, Bodirsky, et al., 2018).

Quorn™ is probably the most well-known example of the successful incorporation of MP in the human diet. This meat substitute based on mycoprotein derived from the fungus *Fusarium venenatum* has a range of over 100 products available in 18 countries (Phillips et al., 2011; Quorn, 2018). Other popular examples include Vegemite and Marmite, two yeast extract pastes that are often consumed as a spread on toast (Vegemite, 2018).

Concerning animal feed, advances have been made by the Canadian company Calysta, producing the MP FeedKind® (Calysta, 2018). Unibio has also entered this market with their UniProtein® (Unibio, 2018). Another interesting example is ValProMic, an MP grown on wastewater from the potato processing industry (Avecom, 2016).

### 1.2 Sustainability

It is projected that the increase in global population, coupled with the increased dietary demands per person, will push the availability of natural resources to their limits. These

non-renewable resources are constrained to their availability on Earth and can thus not be augmented (Flachowsky et al., 2017). The two main restrictions for the required food production are the agricultural land area and water availability, as projections for 2050 show that the planetary boundaries - the environmental limits for safe human interference - will be exceeded for both if the current resource usage does not change (Steffen et al., 2015). Following the current scenario, cropland usage will exceed the planetary boundary by 70%, while freshwater usage will be 50% higher than the limit (Springmann et al., 2018).

Roughly 12% of the land surface has already been converted to cropland, not much lower than the safe limit of 15% of the surface area (Pikaar et al., 2017; Rockström et al., 2009). The upper limit is almost reached, thus allowing little room for further expansion. The arable land area is not only finite, but the growing population results in a continuous diminution of the available area for cultivation per person (Flachowsky et al., 2017). Since the land surface cannot be enlarged, it has to be used more efficiently in order for each person to continue eating enough nutritious food.

Traditional agriculture requires considerable amounts of fresh water, with irrigation accounting for about 70% of the global fresh water usage (Pikaar et al., 2017). Water footprints for protein-rich plant-based products range from 2.4 $m^3$/kg for soy to 4.1 $m^3$/kg for pulses, but increase substantially for animal production, with water needs up to 15.4 $m^3$/kg for beef (Mekonnen et al., 2010). The planetary boundary for environmentally responsible water usage was estimated at 4,000 $km^3$/year (Rockström et al., 2009; Steffen et al., 2015). In 2013, beef production accounted for about a quarter of this amount (FAO, 2013b). As meat consumption is increasing, these water-intensive products will clearly pose a threat to the future fresh water supply (FAO, 2013b).

Introducing MP as a high quality protein could alleviate the stress on these two critical factors. Contrary to livestock, there is no need for arable land to grow these organisms and produce protein. An industrial production site would be sufficient since the cultivation can be performed in compact bioreactors (Cumberledge et al., 2016). Reactor-based MP production also requires substantially less fresh water. A water usage of only 0.005 $m^3$/kg is recorded, which can be even further reduced by recycling the water (Pikaar et al., 2017).

Moreover, micro-organisms demonstrate fast growth with a doubling time of several hours whereas animals double their weight in a time frame of multiple weeks (Goldberg, 1985). This characteristic can be attributed to their high nutrient efficiency, contrary to traditional agriculture, which is characterised by low protein conversion efficiencies and hence considerable losses (Goldberg, 1985; Shepon et al., 2016).

Protein efficiency can be expressed by the amount of nitrogen in the product as opposed to the nitrogen supplied, since protein is the only macronutrient that incorporates this component (carbohydrates and fatty acids do not include nitrogen). The nitrogen cycle starts with the production of ammonia in the Haber-Bosch process and is then introduced in agriculture through ammonia containing fertilisers (Zimdahl, 2015). However, already 50 to 70% of the nitrogen supplied to the soil is lost through runoff and cannot be taken up by crops (Masclaux-Daubresse et al., 2010). The nitrogen that actually ends up in the plant and is available for human consumption, is thus considerably lower than the original amount that was applied. This leads to an overall efficiency of only 14% of initially supplied Haber-Bosch nitrogen available for human consumption (Figure 1) (Pikaar et al., 2017). Conversion efficiencies during animal production are even lower, with only 2-3% of feed nitrogen ending up in beef (Shepon et al., 2016). At the end of the animal production chain, only 4% of the original nitrogen is retained in the end product and available for human consumption (Figure 1) (Pikaar et al., 2017).

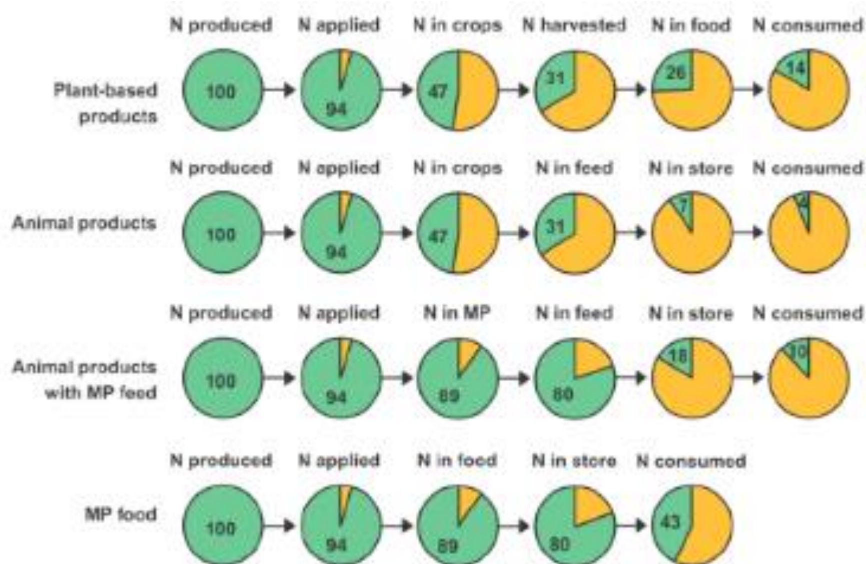



*Figure 1. Haber-Bosch nitrogen efficiency through different protein production pathways. Green represents the fraction of nitrogen retained in the product (Pikaar et al., 2017).*

With increasing agricultural activity to sustain the world population, these losses will only rise. Global nitrogen losses to the environment are estimated to grow by 70% by 2050, while nitrogen fertiliser demand is expected to increase with an additional 50% (Pikaar et al., 2017; Sutton et al., 2013). It is clear that this practice is ultimately not sustainable.

By implementing reactor-based MP production, nitrogen losses would be 3 to 10 times lower than conventional agriculture (Pikaar, Matassa, Rabaey, et al., 2018). Nitrogen usage efficiency in reactors is improved to almost 100% as leaching, runoff or volatilisation

of nitrogen can be almost completely avoided. Additionally, nutrient dosage and consumption can be well monitored, allowing for even more effective nutrient usage and minimal losses (Pikaar, Matassa, Rabaey, et al., 2018).

Another benefit of reactor-based protein production is the independency of climate and weather (Matassa, Boon & Verstraete, 2016; Volova et al., 2010). As the temperature continues to rise due to the global warming effect, traditional agriculture will become continuously more challenging because of increased draught and a varying climate (Jalota et al., 2018). A less climate sensitive food production is thus a valuable characteristic.

To further reduce the impact of the MP production process, the feedstocks for the micro-organisms to grow on should be carefully selected. Industrial or agricultural waste streams still contain valuable nutrients on which the organisms can grow. Apart from valorising these side streams, that would otherwise be disposed and afflict the environment, this practice results in a more competitive product due to the low feedstock price (Najafpour, 2015). Examples include fruit and vegetable processing residues but also more chemical streams such as crude oil, acetic acid and methanol (Garg et al., 1980). This wide array of potential feedstocks also shows the versatility and flexibility of microbial biotechnology (Goldberg, 1985).

In case direct application of waste streams is not possible, these can still form the basis for conversion into suitable substrates for microbial production. For example, acetic acid and formic acid can both be produced by recovery of $CO_2$, by biological and chemical conversion, respectively (Alper et al., 2017; Kumar et al., 2018; Ruiz-Valencia et al., 2019). As $CO_2$ is a known greenhouse gas, diminishing the $CO_2$ levels in air could retard the effects of global warming. Incorporating these recovered compounds in the micro-organisms' feedstock can thus further increase the sustainability of the final product.

Next to being a potential application for recovery of $CO_2$, greenhouse gas emissions are close to zero for MP production, a significant difference to conventional animal-based protein sources (Pikaar, Matassa, Bodirsky, et al., 2018). On average, the global emission intensity for all livestock commodities is 41 kg $CO_2$ equivalents per kg edible protein, although this varies greatly depending on the production system and type of product (Herrero et al., 2013). For example, beef production even mounts up to almost 300 kg $CO_2$ equivalents per kg edible protein, the highest emissions across all products (FAO, 2013c).

Other environmental benefits worth mentioning include the lack of pesticide or fungicide use, in contrast to crop cultivation, since these products deteriorate the environment (Volova et al., 2010).

### 1.2.1 Microbial protein in feed for more sustainable animal-derived protein production

Utilising MP for animal feed, either as an additive to enhance protein quality or as a complete protein replacement, appears to be promising. Especially since conventional high protein feed ingredients such as fishmeal and soybean meal do not have a great reputation when it comes to sustainability.

Fishmeal, which is frequently used as feed in aquaculture, is obtained by drying fish or fish waste (Windsor, 2001). This means that the industrial production of fish relies heavily on other fish, which is ultimately not sustainable. The global overfishing problem has only intensified the concern for the use of fishmeal (IFFO, 2017). To preserve the low fish stocks, fishmeal is being substituted by crop-based feed ingredients with increasing frequency, but by using water- and land-intensive crops, this is merely a shift of the problem (Cumberledge et al., 2016; World Resources Institute, 2013). Additionally, these crops and many fish species used for fishmeal production can be consumed as part of a human diet, and therefore this practice raises a food/feed competition (Cumberledge et al., 2016; Flachowsky et al., 2017).

Soybean meal has been criticised due to the correlation between soybean cultivation and deforestation of the Amazon in order to enlarge the limited agricultural land area (P. D. Richards et al., 2012). These land usage changes are linked with a loss in biodiversity and have a significant impact on the soil and vegetation, which can increase $CO_2$ emissions (Bringezu et al., 2014).

If MP were to be incorporated in animal feed as a substitution for these conventional high protein ingredients, protein efficiency would be increased to 10% consumable nitrogen of the original Haber-Bosch product. The nitrogen losses during different agricultural pathways are illustrated in Figure 1 (Pikaar et al., 2017).

However, a full substitution of high protein feed ingredients for MP is not yet feasible. Depending on social, economic and technological development, MP could replace 10 – 19% of conventional feed protein by 2050 (Pikaar, Matassa, Bodirsky, et al., 2018). This evolution will alleviate the stress on critical environmental factors: cropland area, cropland nitrogen losses and agricultural greenhouse gas emissions can be decreased by 6%, 8%, and 7%, respectively (Pikaar, Matassa, Bodirsky, et al., 2018).

Altogether, the addition of MP in feed could be a sustainable solution. Unlike crops or fish, there is no competition with the human food chain and the natural resource requirements are far lower than the traditional feed ingredients. However, this scenario still relies on inefficient animal production. If MP were to be introduced in the human food chain, this

could even further decrease the nutrient losses and environmental impact of the feed/food chain (Pikaar et al., 2017)

### 1.2.2 Microbial protein in food

The incorporation of MP as a protein source in the human diet, as opposed to in animal feed, would maximise its benefits. The scenario of an MP-based diet would dramatically increase nitrogen efficiency to 43%, which is a tremendous improvement compared to traditional animal production (4%) or the use of MP in feed (10%) (Figure 1) (Pikaar et al., 2017).

However, this scenario is not practicable in the near future. While some MP products are already available on the market (e.g. Quorn™), these only make up a small percentage of sales and are more a niche market (Matassa, Boon, Pikaar, et al., 2016). Broad acceptance of MP consumption would first have to be established in order to launch MP as a full-fledged protein source in the human diet. Additionally, several regulatory and safety barriers hamper fast evolution in this field.

## 1.3 Nutritional value

Protein can account for up to 75% of dry microbial biomass in a reactor with ideal conditions, although average values are lower (Pikaar et al., 2017). Additionally, this concentration can vary depending on the micro-organism used for protein production. An overview of the biomass composition for different groups of micro-organisms is given in Table 1.

*Table 1. Composition of several micro-organisms (% of dry mass) (Anupama et al., 2000; Najafpour, 2015)*

| Component | Yeast | Bacteria | Fungi | Algae |
|---|---|---|---|---|
| Protein | 45–55 | 50–65 | 30–45 | 40–60 |
| Nucleic acid | 6–12 | 8–16 | 7–10 | 3–8 |
| Fat | 2–6 | 1.5–3 | 2–8 | 7–20 |
| Ash | 5–9.5 | 3–7 | 9–14 | 8–10 |

Biomass from bacterial origin shows the highest protein content and a favourable amount of unsaturated fatty acids, making it an excellent source for MP (Aggelopoulos et al., 2014; Ritala et al., 2017). The protein content is not only very high, it is also of good quality with a favourable amino acid composition for feed and food applications. Compared to conventional animal feeds, the composition of MP from bacterial origin is similar to

fishmeal, while yeast protein resembles the soy amino acid distribution (Figure 2) (Najafpour, 2015). Nevertheless, different animal species require varying amino acid compositions, so one single MP product will not ensure sufficient essential amino acid intake for all (Goldberg, 1985). Administering MP from various micro-organisms and in various ratios might resolve this issue.

The amino acid analysis for MP as part of human nutrition is equally promising, delivering sufficient amounts of essential amino acids, as compared to the FAO protein scoring pattern (Figure 3). (FAO, 2013a; Matassa, Boon & Verstraete, 2016). This protein scoring standard is based on the human requirements for essential amino acids to quantitatively compare protein quality. The milk protein casein, often used as a reference protein, has also been included for comparison (Volova et al., 2010).

Most amino acids in MP, whether from bacteria or yeast, are present in concentrations above the FAO threshold, although a limiting factor is the sulphur-containing amino acid content in protein from HOB. The similarity between MP and animal protein (casein) demonstrates the high biological value of the product (Volova et al., 2010).

Amino acid distributions of the MP might seem very close to those of the conventional protein sources, but varying protein content between these products will affect the final essential amino acid content (Matassa, Boon & Verstraete, 2016).

However, it should be noted that information about protein content and composition does not allow for a comprehensive estimation of the nutritional value. Of course, a product should supply sufficient essential amino acid, but a decisive factor is the bio-availability of these nutrients. Amino acid absorption in the digestive tract is likely lower than the original content, affecting the biological value of the protein. Even if a protein shows an outstanding amino acid distribution and content, its nutritive quality won’t be high if the nutrients cannot be absorbed during digestion. Therefore, availability of the proteins to proteolytic enzymes should be taken into account as well (FAO, 2013a; Volova et al., 2010).

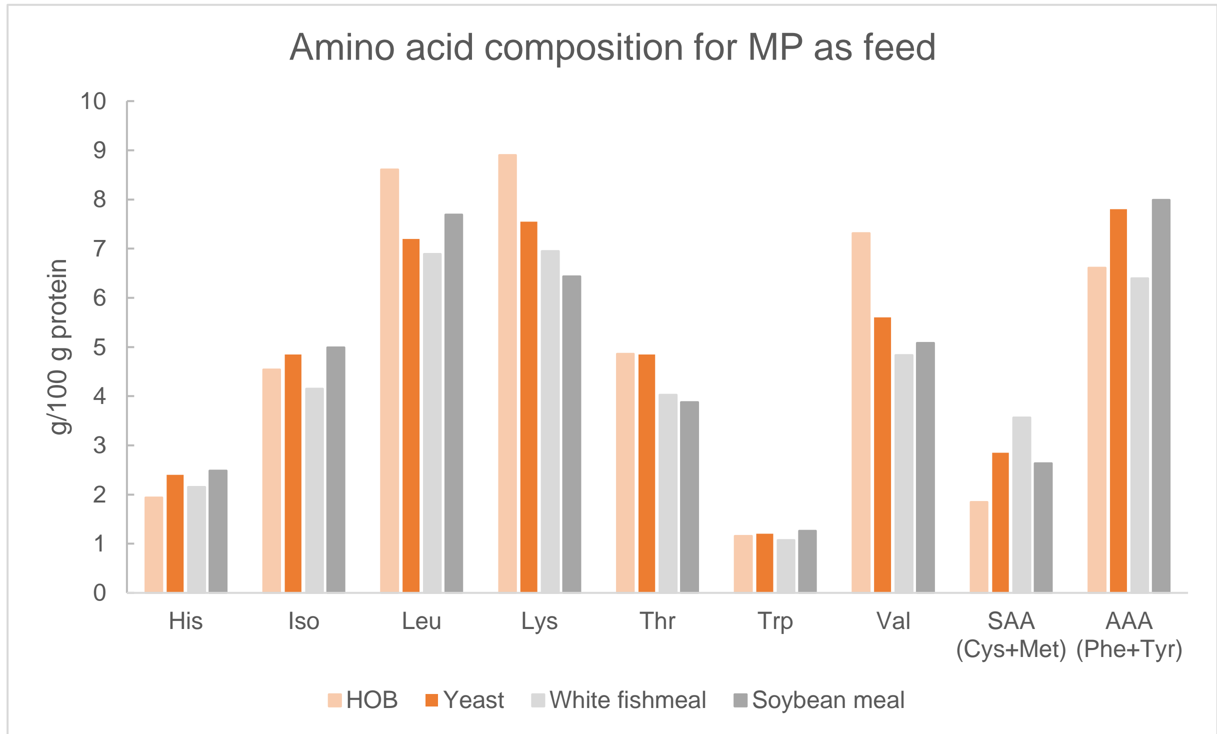



*Figure 2. Comparison of essential amino acids for microbial protein from HOB (C. necator) and yeast (S. cerevisiae) to fishmeal and soybean meal. His=Histidine, Iso= Isoleucine, Leu= Leucine, Lys= Lysine, Thr= Threonine, Trp= Tryptophan, Val= Valine, SAA= Sulphur containing amino acids, AAA= Aromatic amino acids. References: Appendix 1.*

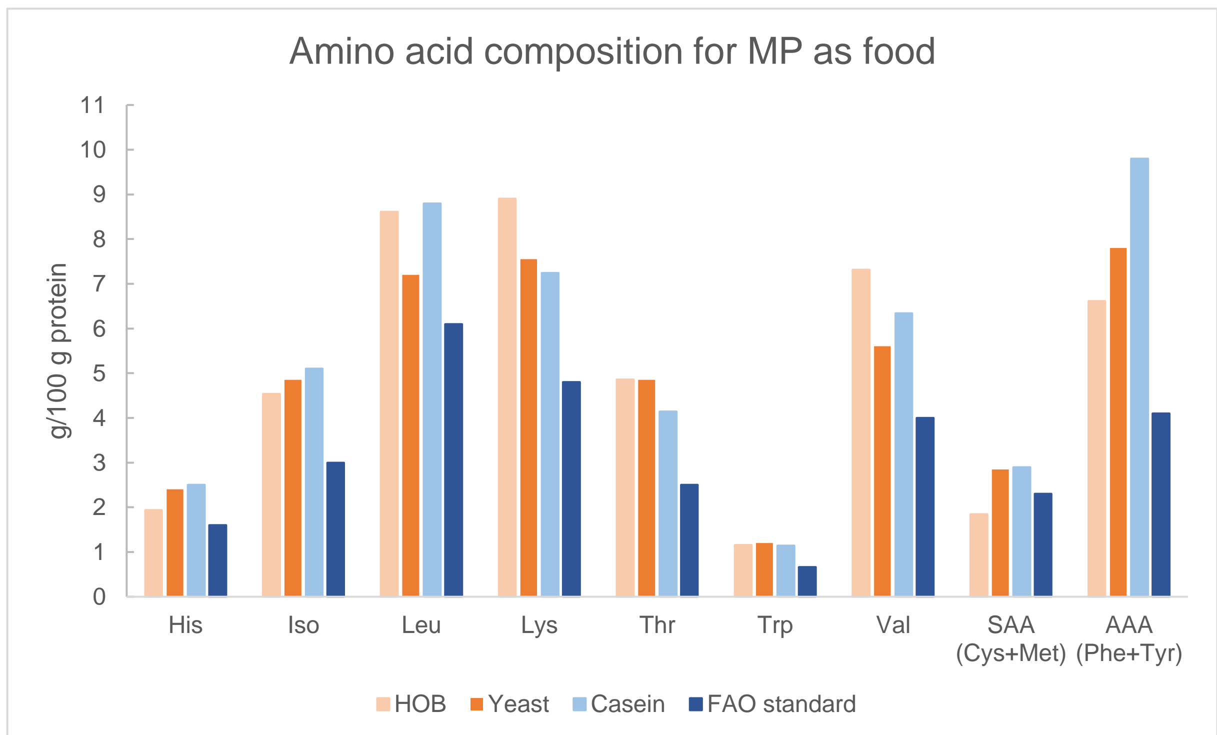



*Figure 3. Comparison of essential amino acids for microbial protein from HOB (C. necator) and yeast (S. cerevisiae) to casein and the FAO protein standard for human essential amino acid requirements. His=Histidine, Iso= Isoleucine, Leu= Leucine, Lys= Lysine, Thr= Threonine, Trp= Tryptophan, Val= Valine, SAA= Sulphur containing amino acids, AAA= Aromatic amino acids. References: Appendix 1.*

Digestion experiments with rats, which are used as a model system for humans and animals, showed that 93% of protein from HOB was absorbed, compared to 99% for the animal protein casein (Calloway et al., 1969). Other trials observed digestibility values ranging from 65 – 96% for various micro-organisms (Anupama et al., 2000). Nitrogen digestibility for fishmeal and soybean meal is 91% and 86% of consumed nitrogen, respectively (International Association of Fish Meal Manufacturers, 1970). Proteins of plant origin usually have a lower availability than animal-derived protein. The digestibility of MP is intermediate to these two groups, which should result in a high nutrient uptake (Volova et al., 2010).

In order to gain a more comprehensive understanding of the protein value, the nitrogen fraction that is effectively utilised after absorption should be assessed as well. The rest of the nitrogen is excreted via urine and thus not actually used. The study by Calloway & Kumar (1969) reported that, once absorbed, no difference in amino acid utilisation was observed between animal and microbial protein. Several MP products have also been used as protein supplements in feed trials, supporting favourable growth and reproduction in animals (Goldberg, 1985). In conclusion, the absorption of the nutrients is the crucial point when evaluating protein quality and biological value (International Association of Fish Meal Manufacturers, 1970).

Besides protein, several other cellular compounds can be of nutritive value such as vitamins, lipids, carbohydrates, polyhydroxybutyrate (PHB), and carotenoids. The oil can be applied as a substitute for vegetable oil or used for biofuel production. PHB is known for its prebiotic effects, offering an alternative to the antibiotic additions in animal feed (Matassa, Boon & Verstraete, 2016).

## 1.4 Flavour

Even though the nutritional value seems promising, the flavour is still a determining factor for the commercial success of the product. It does not only play a role in consumer preference: animals can be just as picky since they use flavour as a warning mechanism to reject or accept food (Kawai et al., 2009; Roura et al., 2017).

Flavour is the sensory experience of food from the chemical senses, mainly a combination of taste and scent (Gibson et al., 2018). The odour plays a dominant role in the sensory experience and thus flavour of a product, allowing for a profound estimation of the flavour through characterisation of the scent (Spence, 2015). This property can be mechanically measured and compared with fragrance databases.

A scent is generated when odour active compounds are volatilised and reach the olfactory system. More volatile molecules generate a more intense smell, which is why odorous

molecules usually have little or no polarity, as these tend to have weaker intermolecular bonds. The fragile bonds are easily broken, allowing the molecule to be released quickly. A higher temperature and lower molecular weight also favour volatility (Gibson et al., 2018).

These volatile organic compounds (VOC) can be detected to form a volatile profile, from which the scent and thus the flavour can be derived. Traditionally, trained panels were established to assess tastes and aromas, but now several aroma sensors or electronic noses (eNose) have been developed to mimic the human olfactory system. The eNoses function by assessing and evaluating the entire aroma profile, rather than selecting a few dominant sensors (Rottiers et al., 2018). However, these sensors only allow for a qualitative evaluation of aroma. To this end, the most recent techniques include gas chromatography to also quantify and determine the compounds responsible for the odour (Wardencki et al., 2013).

The novel odour-sensing techniques allow for an objective evaluation of flavour, as the human system is quite subjective when it comes to flavours. On the other hand, the technological sensors are not nearly as sensitive, so that taste or odour panels are still valuable in the sensory science (Wardencki et al., 2013). A combination of these two methods is likely to yield the best results.

The flavour of fermented foods has been thoroughly studied through the use of various techniques (Zhao et al., 2016). Microbial fermentation has been applied to preserve food and transform taste for thousands of years and many fermented foods, such as yoghurt, beer, and salami, are a part of the human diet to this day. These foods are characterised by a rich and complex flavour, derived from small peptides and free amino acids, generated by microbial proteolysis and amino acid conversions. The actual taste of the peptides, and whether they are taste-active, depends on amino acid composition and sequence (Zhao et al., 2016).

However, the flavour of MP has not been assessed with these techniques. Although the biomass and amino acid composition for several micro-organisms has been determined, this does not allow to make accurate assumptions on the flavour of MP (Najafpour, 2015). As flavour is influenced by many different organic compounds and their interactions, a sole evaluation of biomass composition would not be sufficient.

## 2. Barriers for the use of microbial protein

MP has already come a long way since the initial research going on in the previous century, although some hurdles still hamper the widespread integration of MP. As with any novel feed or food, introduction in Europe is strictly regulated and requires a thorough safety study prior to commercialisation (Regulation (EU) 2015/2283). In the case of MP, a few concerns have already been established.

The high protein content of bacterial MP may account for a promising nutritional value, but these organisms also contain undesirably high concentrations of nucleic acids, as part of their RNA. After ingestion of these molecules, the purine nucleobases that make up the nucleic acids are broken down, with the formation of uric acid. The uric acid cannot be further metabolised in humans due to lack of the uricase enzyme and starts to accumulate (Anupama et al., 2000). The accumulation of the low soluble uric acid then causes the formation of urinate crystals in several tissues, which can lead to the formation of gout and kidney stones (El Ridi et al., 2017). These effects can already be seen after consumption of more than 2 g nucleic acids per day (Anupama et al., 2000).

The recommended dietary intake for protein is set at 0.75 g per kg body weight per day, or 51 g per day for an average adult (World Resources Institute, 2016a). If someone were to consume this amount of MP as their sole protein source, excessive daily nucleic acid intake up to 12.5 g is possible, significantly higher than the 2 g/day threshold, assuming a protein content of 65% and a nucleic acid content of 16%. It is clear that this high purine content is a major drawback and significantly diminishes the nutritional value of MP for humans. Animals consuming MP through their feed would not face these problems as they possess an active uricase enzyme, converting uric acid to the more soluble allantoin (El Ridi et al., 2017; Rumsey et al., 1991).

The high nucleic acid content should thus be addressed if the product is intended for human consumption. It would seem more convenient to obtain biomass with a lower nucleic acid content, but as protein production is closely related to RNA content, this would likely result in lower protein concentrations (Goldberg, 1985). Therefore, nucleic acid removal should be performed during downstream processing, which adds to the intricacy of this production step, as explained below.

Food grade MP production would also require rigorous control to avoid contamination by pathogens and toxin producing organisms (Anupama et al., 2000). If the micro-organisms are grown on waste streams, the quality of the feedstock could be an added hazard. In this case, the main concern would be the presence of heavy metals or other harmful compounds (Ritala et al., 2017). Other safety considerations include potential allergy

symptoms, as proteins are commons triggers for immune reactions (Pali-Schöll et al., 2018).

Mutagenic properties of MP grown on methanol have already been evaluated in vivo, showing no mutagenicity by statistical analysis (Anupama et al., 2000). Nevertheless, more in depth testing is required to provide an overall risk assessment of MP consumption, both for animals and humans. These control measures are not only vital to care for consumers' health, they are also required by the European novel food legislation (Regulation (EU) 2015/2283).

European regulations also require approval prior to the application of a novel product, both for feed and food (EFSA, 2018). Although MP of some species has already been approved as a feed additive, an application has to be submitted for approval for new species as each organism has different properties (EFSA, 2018; Pikaar, Matassa, Bodirsky, et al., 2018; Regulation (EU) No 68/2013). Recently, biomass from the yeast *Yarrowia lypolitica* has also been approved as a supplement for human nutrition (Regulation (EU) 2019/760).

Although this official recognition is a big step forward, societal acceptance still needs to be achieved. As seen with genetically modified organisms (GMO) and edible insects, food neophobia is a major hurdle in consumer adoption, no matter how beneficial the nutritional value is (Anupama et al., 2000; Shockley et al., 2014). Rational facts alone cannot persuade hesitant consumers. Take insects for example, several studies have been performed assessing the sustainability and nutrient content, concluding that they make for a suitable protein source (Shockley et al., 2014). Yet, customers in Western countries are turned off by the idea of consuming insects (Menozzi et al., 2017).

However, MP might be adopted more easily since microbial-based food products such as Quorn™ are already available. Baker's yeast and fermented products are staple foods in our diet, possibly easing the path for an MP breakthrough (Pikaar, de Vrieze, et al., 2018).

Of course, these mental barriers are less of an issue in the feed industry, but producers or farmers still have to be convinced to provide MP to their livestock. Widespread acceptance, both in the feed and food industry, first has to be established in order to reach a sufficient demand and a profitable product (Pikaar et al., 2017).

Depending on the production pathway and substrates, manufacturing costs of MP are still significantly higher compared to current market prices of conventional feed ingredients such as soybean meal and fishmeal (Anupama et al., 2000; Pikaar, de Vrieze, et al., 2018). On the other hand, utilisation of waste streams can diminish expenses and commercially available methane-based MP indicates economic viability (Matassa, Boon, Pikaar, et al., 2016; Pikaar et al., 2017).

Another drawback could be the intricate and costly downstream processing. The cells are washed to remove any potential harmful substances and then harvested by a solid-liquid separation method (Anupama et al., 2000; Calloway et al., 1969). However, the low biomass concentration (typically around 5-10 g $L^{-1}$) and small cell size make this an expensive and energy intensive process, suggesting the need for optimisation (Goldberg, 1985). Several harvesting procedures have been proposed, of which flocculation, centrifugation, and filtration seem most suitable (G. Singh et al., 2018; M. Singh et al., 2013).

Further, sufficient measures should be taken to ensure that the biomass is not viable before consumption to avoid microbial growth during digestion (Calloway et al., 1969). This will also substantially diminish the risk of pathogenic contamination (Goldberg, 1985). Lastly, the biomass is dried, a crucial step in the final product development (Matassa, Boon & Verstraete, 2016).

## 3. Hypothesis

In order to market MP to its full potential, it is vital to assess the protein content and flavour, but more importantly, how these factors are affected. As flavour and composition of food products varies constantly, it seems likely that MP quality might differ similarly (Wang et al., 2013). Influencing factors might range from cultivation conditions to the studied microbial species and downstream processing. These factors are analogous to conditions affecting traditional food products, as these vary just as much by origin, species, and downstream processing .

Cultivation conditions, such as the provision of a specific carbon source, could affect protein content and flavour volatiles. Additionally, different carbon substrates can affect the growth rate, which is known to influence protein content, although it is not known how this impacts the flavour (Volova et al., 2010).

It was also hypothesised that the protein content in the cell does not remain constant throughout the growth of the micro-organism. Since higher growth rates increase protein synthesis, the protein levels in the micro-organisms (percentage of total suspended solids, %TSS) were assumed to be the highest during the exponential growth phase, when maximal growth speed is reached (Volova et al., 2010). This growth rate dependency can be attributed to the large part of ribosomal RNA present in the bacterial RNA (Bremer et al., 2008). Because of this link between the protein-producing ribosomes and the amount of bacterial genome, an increase in ribosomes also accounts for a gain in ribosomal RNA.

As the fast cell division requires enormous RNA production, the abundance of ribosomes increases at higher growth rates, thus allowing for more protein production (Bremer et al., 2008; Klumpp et al., 2009).

Next, different organisms are expected to result in a product with different characteristics. Varying protein contents and amino acid distributions have been observed among micro-organisms, which could also account for a flavour difference. Additionally, the organisms might produce other odorous compounds, such as volatile fatty acids, possibly further differentiating the flavour.

Thus, the goal of this research was to evaluate the influence of the micro-organism and the carbon source on both protein content and flavour, and the influence of the growth phase on protein content.

# III. MATERIALS AND METHODS

## 1. Microbial cultures

*Cupriavidus necator* (strain LMG 1199), previously known as *Alcaligenes eutropha* and *Ralstonia eutropha*, was included in the experiments to be studied as a pure culture. This organism has been studied extensively for the production of MP, which made it a suitable candidate for this research (Chee et al., 2019).

The enrichments were obtained through an enrichment process starting from the ValProMic microbiome, a culture grown on potato processing waste streams for the production of MP (Avecom, 2016). This initial culture was first grown on Nutrient broth (section 2.1), and served as the inoculum for two enrichment procedures. In each series, the organisms were supplied with a different carbon source, acetate and formate, yielding two diverse enrichments that were accustomed to consume the provided feedstocks. The goal was to select for fast-growing and settling micro-organisms.

From these resulting cultures, bacterial strains were isolated by dilution-to-extinction (Yan et al., 2015). A 1:10 inoculum dilution series, up to a dilution factor $10^{10}$, was set up in sterile 96-well plates containing the growth medium and the respective carbon substrate. After two days of growth, the most diluted sample that showed microbial growth was plated, from which a single colony was selected and plated three more times. These isolates were also included in the batch experiments.

The acetate isolate was identified (section 5.4) as *Acinetobacter sp.* and the formate isolate was *Paracoccus sp.*

## 2. Media composition

### 2.1 Pre-cultures

The *C. necator* pre-cultures, as well as the inoculum for the enrichments, were grown on nutrient broth (Carl Roth), at a concentration of 8 g $L^{-1}$.

The enrichments on acetate and formate were conducted using the same AMS medium as employed in the batch growth experiments (section 2.2) In order to avoid acid evaporation, as well as extreme pH shifts, acetate and formate were supplied in the form of sodium salts. For sodium formate, the same dosage as in the batch experiments (3.40 g $L^{-1}$) was applied, but the concentration for sodium acetate was increased to 4.10 g $L^{-1}$,

so that both carbon sources had a final concentration of 50 mM. The goal of the increased amount of acetate was to allow for longer growth, so that the stationary phases would be attained in a similar time period in both enrichments.

The strains isolated from the enrichments were grown on the same medium as their respective enrichments.

The compositions outlined here are those for the liquid media. For the solid agar plates, 15 g $L^{-1}$ of agar (Carl Roth) was added.

## 2.2 Batch experiments

The batch experiments were conducted on ammonium mineral salts (AMS) medium with added vitamins (from DSMZ medium 81). The composition is given in Table 2 and was employed for all species (DSMZ, 2011).

Acetate and formate were added in the form of their sodium salts, at a concentration of 1.03 g $CH_3COONa$ $L^{-1}$ and 3.40 g HCOONa $L^{-1}$ respectively, in order to consistently provide 0.81 g COD $L^{-1}$. How these carbon levels were determined is explained in section 3.

*Table 2. Composition of AMS medium with vitamins*

| **AMS medium** | | | | | |
|---|---|---|---|---|---|
| Mineral salts | | | Phosphate buffer | | |
| Compound | Value | Unit | Compound | Value | Unit |
| $MgSO_4$ x 7 $H_2O$ | 1 | g $L^{-1}$ | $Na_2HPO_4$ x 12 $H_2O$ | 0.717 | g $L^{-1}$ |
| $NH_4Cl$ | 0.5 | g $L^{-1}$ | $KH_2PO_4$ | 0.272 | g $L^{-1}$ |
| $CaCl_2$ x 2 $H_2O$ | 0.15 | g $L^{-1}$ | | | |
| FeNaEDTA | 0.05 | mg $L^{-1}$ | | | |
| Trace elements | | | Vitamins | | |
| Compound | Value | Unit | Compound | Value | Unit |
| $Na_2EDTA$ x 2 $H_2O$ | 0.5 | mg $L^{-1}$ | Riboflavin | 0.005 | mg $L^{-1}$ |
| $FeSO_4$ x 7 $H_2O$ | 0.2 | mg $L^{-1}$ | Thiamine-HCl x 2 $H_2O$ | 0.025 | mg $L^{-1}$ |
| $H_3BO_3$ | 0.03 | mg $L^{-1}$ | Nicotinic acid | 0.025 | mg $L^{-1}$ |
| $CoCl_2$ x 6 $H_2O$ | 0.02 | mg $L^{-1}$ | Pyridoxine-HCl | 0.025 | mg $L^{-1}$ |
| $ZnSO_4$ x 7 $H_2O$ | 0.01 | mg $L^{-1}$ | Ca-pantothenate | 0.025 | mg $L^{-1}$ |
| $MnCl_2$ x 4 $H_2O$ | 0.003 | mg $L^{-1}$ | Biotin | 0.05 | µg $L^{-1}$ |
| $NaMoO_4$ x 2 $H_2O$ | 0.003 | mg $L^{-1}$ | Folic acid | 0.1 | µg $L^{-1}$ |
| $NiCl_2$ x 6 $H_2O$ | 0.002 | mg $L^{-1}$ | $B_{12}$ | 0.5 | µg $L^{-1}$ |
| $CuSO_4$ x 5 H2O | 2.5 | mg $L^{-1}$ | | | |

## 3. Carbon sources

To study the impact of the feedstock on protein content and flavour, acetate and formate were selected as the two most suitable carbon sources because of their potentially sustainable production pathway (section II.1.2). However, organic acids can inhibit microbial yields at higher concentrations, so the non-limiting concentrations had to be determined first (Grunwald et al., 2015; Marudkla et al., 2018). To this end, growth experiments were conducted with varying concentrations of acetate or formate, i.e. 5.40, 10.9, 16.3, 32.9, 43.4, 65.2, 109, 130 and 173.8 mM. The growth of *C. necator*, the enrichment cultures, and the isolates was then monitored in a 96-well plate through the use of a plate-reader (Tecan Infinite M200 Pro). Absorbance at 600 nm was measured every 15 minutes, following a cycle of continuous orbital shaking at 180 rpm for 14:45 minutes and 15 seconds rest before the measurement. The final working volume in the wells was 0.2 mL, after 10 v% inoculation.

From the obtained growth curves, the growth rate, as obtained in section 7.1, served as the basis to select a suitable acid concentration, which was then applied for all species. The levels for the acetate and formate were calculated to deliver the same chemical oxygen demand (COD) to the organisms.

## 4. Growth conditions

The goal of this research was to determine the influence of the carbon source and the growth phase on the protein content, as well as the influence of the growth phase and choice of micro-organism on the flavour. These parameters were assessed in batch experiments, where growth was monitored by OD and pH.

### 4.1 Pre-cultures: cultivation and inoculation

#### 4.1.1 Pure cultures

The strains were grown on agar plates at 28 °C and stored at 4 °C after incubation to sufficient growth. Each micro-organism was grown on a separate medium to tailor the nutrients to its needs, as lined out in section 2.1.

The following procedure was performed to cultivate each pre-culture. A single colony was transferred from a nutrient rich agar plate to 5 mL of the corresponding liquid medium and incubated for 24-48 hours at 28 °C. After sufficient growth, this biomass was used to inoculate a falcon tube with 25 mL fresh liquid medium (90 v% medium + 10 v% inoculum). The biomass was incubated for another 24 hours at 28 °C, this time with constant shaking

at 120 rpm. This culture was transferred once again to an Erlenmeyer (90 v% medium + 10 v% inoculum) and incubated at 28 °C at 120 rpm. This final pre-culture was used as inoculum for the batch experiments. The procedure is also presented in Figure 4.

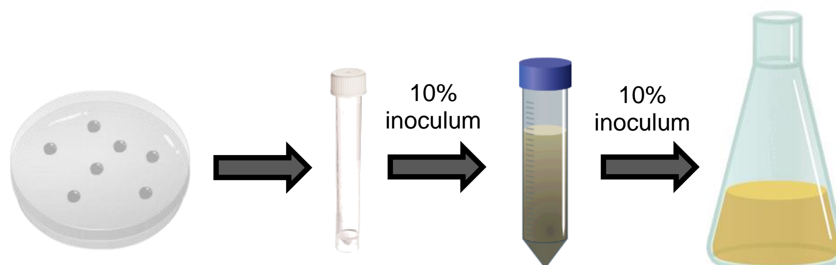



*Figure 4. Inoculation procedure for calibration curve.*

Before inoculation, the organisms were subjected to a washing step with 0.08 M phosphate buffer to remove any traces of the nutrient-rich medium. The aim of this step was to ensure that any growth observed during the experiment can be solely attributed to the delivered nutrients and is not induced by traces of the medium from previous cultivations.

The washed culture was then concentrated or diluted to reach a set OD value (Table 3) in order to standardise the initial biomass content for separate experiments. This OD value was defined for a setpoint cell count of ca. $5 \cdot 10^8$ cells $mL^{-1}$, as obtained by flow cytometry beforehand. The flasks were inoculated at a 10 v% level to achieve initial cell concentration of ca. $5 \cdot 10^7$ cells $mL^{-1}$.

*Table 3. Setpoint OD values for the pre-cultures used as inoculum for the batch experiments.*

| Strain | *C. necator* | *Acinetobacter sp.*<br>*Paracoccus sp.* | Enrichments |
|---|---|---|---|
| OD value | 1.37 | 1.36 | 1.40 |

### 4.1.2 Enrichments

The ValProMic cultures were first subjected to an enrichment process to yield cultures accustomed to acetate and formate, respectively. The enrichments were executed under non-sterile conditions in 500 mL Erlenmeyers (working volume of 250 mL) at 28°C shaking at 120 rpm. Every other day, a flask with fresh medium was inoculated (10% of final volume) using the previous culture. In between these inoculation days, the microbiomes were supplied with their respective carbon source. Growth was monitored by daily $OD_{600}$ measurements after vigorous stirring. After repeating this process for 9 days, the biomass was centrifuged and resuspended in fresh medium on day 10. The procedure then continued on as before.

After 17 days, the enrichment procedure was altered to start selecting for settling biomass. Following 30 minutes of settling time, 50 mL of supernatant (20 v%) was removed daily and replaced with an equal volume of fresh medium. OD was now measured in the supernatant, after the addition of the new medium. This procedure was carried out daily for 7 days, after which the enrichments were applied as inoculum for the respective batch experiments.

Before inoculation, the obtained cell suspensions were subjected to the washing step, after which the same procedure was used as for the pure cultures. The setpoint OD value before inoculation is also included in Table 3.

## 4.2 Batch experiments

All batch experiments were performed in 1 L Erlenmeyers, with a working volume of 400 mL. These flasks were incubated at 28 °C and at constant shaking of 120 rpm. All experiments were performed in triplicate, with 1 blanc containing distilled water instead of inoculum.

# 5. Biomass characterisation

## 5.1 Sampling

### 5.1.1 Pure cultures

Samples (2 mL) were taken every 2 to 3 hours, after which the optical density (OD) at 600 nm as well as the pH were measured to monitor the growth. OD measurements were conducted in 4 mL cuvettes with a Spectronic$^{TM}$ 200 spectrophotometer (ThermoFisher Scientific). These OD results were then translated to the biomass concentration via predefined OD-TSS calibration curves. The method that was used to obtain these calibrations is explained in section 5.2.1.

The original sample volume of 2 mL was divided in two 1.5 mL Eppendorf's, each containing 950 µL, and were centrifuged for 5 minutes at 14,000 rpm (20,817 rcf). A supernatant volume of 850 µL was carefully removed from both Eppendorf's to not disturb the formed pellet and added to the residual original sample in the 2 mL Eppendorf. This combined supernatant was preserved at -20 °C for the carbon source concentration analyses, as explained in section 5.3.

The biomass pellet was then washed with 850 µL 0.08 M phosphate buffer by centrifugation at 14,000 rpm (20,817 rcf) for 5 minutes, after which the same volume of

supernatant was discarded. The obtained pellet was stored at -20 °C for the protein analyses, see section 5.3.

### 5.1.2 Enrichment cultures

Unlike the experiments with the pure cultures, calibration curves could not be established for the enrichments. As the microbiome composition could change over time due to the multitude of varying bacterial strains, the OD might not have been reliable as a biomass concentration estimate. To this end, additional sampling volumes were required for the TSS measurements.

Thus, a volume of 25 mL was collected from every replicate during each sampling round. After OD and pH measurements, 2 Eppendorf's were prepared for every replicate, containing 1 mL of sample each. The volume of supernatant removed after centrifugation was 900 µL. The washing step was also executed with 900 µL. The rest of the procedure was analogous to the pure cultures. The remaining sample volume was used for the determination of biomass concentration, as explained in section 5.2.2.

## 5.2 Biomass concentration

The concentration of the biomass was expressed as TSS (mg $L^{-1}$). Volatile suspended solids (VSS, mg $L^{-1}$) are also commonly used as an estimate for microbial biomass, but given that the final MP product will contain the microbial biomass as is, and not only the biodegradable part, it is more appropriate to express the results as a function of TSS in this case.

### 5.2.1 Pure cultures: calibration curves

For the pure cultures, the biomass content was based on the OD by means of a calibration curve. The relationship between OD and TSS was established for each culture, to avoid having to analyse the biomass concentration for each sample separately. The obtained calibrations are presented in Appendix 2.

The procedure performed to obtain each pre-culture was the same as for the inoculum of the batch experiments. The organisms were also subjected to a washing step with 0.08 M phosphate buffer to remove any remnants of the growth medium, before resolving the biomass in AMS. By dissolving the obtained biomass in the AMS medium, the conditions of the batch growth experiments were mimicked as best as possible.

Starting from this AMS-biomass solution, a dilution series was set up for the OD measurements. Series of 9 samples (25 mL each) with OD values ranging from 0.1 to 2 were obtained by concentration/dilution of the biomass-AMS solution.

The measurements for TSS and VSS were performed in triplicate, following Standard Method 2540D (TSS) and 2540E (VSS) (APHA, 2017). Weight difference of the clean filter and after filtering 5 mL of the sample and drying at 105 °C yielded the TSS, while the difference in filter weight after incinerating at 550 °C allowed for the VSS calculations.

For *C. necator,* a different route had to be taken as the filters did not withhold the cells sufficiently. Therefore, a sample volume of 5 mL was dried at 105 °C yielding the total solids (TS) in a crucible and incinerated at 550 °C to result in the volatile solids (VS). These were also performed in triplicate.

### 5.2.2 Enrichment cultures

TSS and VSS measurements for the enrichments were performed during sampling of the batch experiments. The method used was the same as described above, only this time the sample volume was 12 mL. Due to the volume limitation, the analysis was not performed in triplicate per replicate but in biological triplicate.

## 5.3 Carbon consumption and protein content

The residual concentration of the carbon sources was estimated from the supernatant by ion chromatography (IC) (930 Compact IC Flex, Metrohm) for the organic acids with a cation exchange column (Metrosep Organic Acids 250/7.8, Metrohm). All samples were filtered through Minisart 0.20 µm pore-size filters (Sartorius) prior to analysis.

Protein content was determined by the Markwell method (1978) with bovine serum albumin (Carl Roth) as a standard.

## 5.4 Identification of isolated strains

In order to identify which organisms were isolated from the acetate and formate enrichments, samples (2 mL) were taken after completion of the batch growth cycle. After centrifugation for 5 min at 14,000 rpm (20,817 rcf), the supernatant was discarded and the obtained pellet was stored at -20 °C for further analysis. DNA was extracted by bead beating with a PowerLyzer instrument (Qiagen, Venlo, Netherlands) in combination with phenol/chloroform (Vilchez-Vargas et al., 2013). The obtained polymerase chain reaction (PCR) amplicons were purified using the innuPREP PCRpure Kit (Analytikjena, Jena, Germany) and identified through Sanger sequencing (LGC Genomics Gmbh, Berlin) with the primer pairs 27F and 1492R (Ludwig, 2007). The sequences were then blasted against National Center for Biotechnology Information (NCBI) database to identify the two isolates.

# 6. Flavour characterisation of the dried product

## 6.1 Sampling

As a sufficient amount of dried sample was required for the eNose analyses, the impact of the growth phase could not be assessed, since the samplings at each time point comprised too small volumes to yield enough product for the eNose. Instead, these samples were prepared after the stationary phase had been reached, so that the influence of the micro-organism and the carbon source could be analysed.

During every batch experiment, an additional 3.6 L of media was prepared for batch growth in 2 L Erlenmeyers with a working volume of 750 mL. Unlike the smaller Erlenmeyers, these flasks were not monitored throughout growth, but as these ran simultaneously, the stationary phase could be derived from the OD measurements of the batch experiments.

The fully-grown biomass was harvested by repeatedly centrifuging, 5 minutes at 14,000 rpm (20,817 rcf), and removing the supernatant to allow for new sample volume. After collecting and concentrating the complete biomass, a washing step with 0.08 M phosphate buffer was included to remove any traces that might influence the aroma profile. The samples were dried overnight at 60 - 70 °C in a drying oven and ground as finely as possible using a mortar and pestle. Lastly, the air was flushed out and replaced by inert Argon gas to avoid any sample deterioration from oxidation during storage (C. Spencer et al., 2001). The obtained samples are displayed in Appendix 4.

Next to the samples grown on acetate and formate from this study, the dried sample of *C. necator* grown on nutrient broth, as well as the industrially produced MP ValProMic (Avecom, Belgium) were included as references for the eNose analysis. This could give an idea whether the samples from this study could potentially be applied in the animal feed industry, where a similar aroma profile could indicate a potentially applicable product

## 6.2 Analytical parameter optimisation

A suitable analysis method for the MP odour fingerprinting was not readily available in the AlphaSoft software (v 12.4, Alpha MOS). Thus, a novel procedure had to be established. The AlphaSoft method database had a pre-set method for powdered Spirulina, which formed the basis for the development of the new approach. The main challenge was the required sample quantity of 2 grams set out by the pre-set method. Given the difficulty of producing this amount of biomass in batch mode, the method had to be adjusted in order to sufficiently differentiate between blanks and samples at a quantity of 0.1 grams per vial. If a lower sample quantity is analysed, the peaks of the chromatogram are likely to be less high and more difficult to separate. This effect was counteracted by adjusting the ratio of

the sampled headspace that is sent to the GC columns and that is discarded, i.e. the split. A split ratio of x indicates that 1 in x parts of the headspace sample is injected in the capillary columns (Agilent technologies, 2015). The lower the split ratio, the higher the ratio of headspace sample being analysed.

A series of 3 methods was established with a split ratio of 0, 1 and 5 mL $min^{-1}$. All other parameters were equal. To ensure the sample quantity allowed for sufficient differentiation, each method was run with 3 varying sample quantities: 0.1, 0.5 and 1 g. The method optimisation tests were executed with dried ValProMic biomass, the inoculum for the enrichments, as obtained from Avecom (Belgium). These results are presented in Appendix 3, after which the split ratio of 5 was chosen for the analysis of all other samples.

Samples were analysed in a random order, with 5 replicates of every sample. Before the first and after the last sample, 2 blanc vials (air) were included, as well as between every 5 samples. The blanc vials were capped in the same room as the vials containing the samples. The vials were capped with PTFE/silicone seals to avoid contamination of external aromas.

## 6.3 eNose operational system

Fingerprints of the volatile compounds were obtained by headspace analysis with a dual fast GC electronic nose (Heracles II, Alpha MOS). The eNose was equipped with an autosampler (Odor Scanner HS 100, Alpha MOS), a cooled Tenax trap, two capillary columns with a flame ionisation detector (FID) at the end of each column. The first column contained a non-polar coating (MXT-5, 5% diphenyl), while the second column enclosed a layer of medium polarity (MXT-1701, 14% cyanopropylphenyl). Both columns had a length of 10 m, an internal diameter of 180 µm and a film thickness of 0.4 µm.

To release the volatile compounds of the dried samples (0.1 g) into the headspace, the vials were first incubated at 50 °C for 20 min in a thermostatic agitator at 500 rpm. After sufficient incubation, 5 mL of the headspace was sampled with a syringe (60 °C) and injected (200 °C) into the GC system at a flow rate of 125 µL $s^{-1}$ for 45 s with hydrogen as the carrier gas (Parker Balston). Before separation in the GC columns, a purge-and-trap system was applied to adsorb and pre-concentrate the volatile compounds. The trap temperature program started at 40 °C for 50 s, after which the temperature was increased to 240 °C in 35 s and then kept at this temperature for another 30 s. The vent of the injector was set at 30 mL/min and the split ratio of the trap at 5 mL $min^{-1}$. The valve (250 °C) controlled the insertion of the volatiles into the columns for separation. The following time-temperature program was applied: the temperature was first held for 2 s at 50 °C, then raised to 80 °C at 1 °C $s^{-1}$, and finally raised up to 250 °C at a rate of 3 °C $s^{-1}$ and held at

this temperature for 21 s. The signals from the two FID detectors (260 °C) were transmitted every 0.01 s, with a total acquisition time of 110 s.

### 6.4 Identification of volatile compounds

An alkane series (C6 to C16) standard solution (Restek) was analysed using the same analytical method as the samples to calculate the Kovats retention indices (RI) based on the retention times. Volatile components were identified by comparing the experimentally defined Kovats RI with the RI found in the AroChemBase library (v 6, Alpha MOS). The sensorial descriptors of each volatile were retrieved from AroChemBase, as well as from literature (Arn et al., 1998; Burdock, 2016).

Given the large number of small peaks, these could not all be assessed. Instead, the 20 peaks that accounted for the largest discrimination power between samples, after removal of the blanks, were selected for further analysis. Additionally, only peaks with an area above 50 were considered. To this end, the volatiles that showed the greatest variance in concentration were assessed, which allowed for the identification of the key components that made each sample unique.

As no designated library for microbial samples was available in AroChemBase, the volatiles linked to each peak/Kovats RI could not be narrowed down, and were quite general, which resulted in extensive list of potential compounds. The final component was then selected as the most occurring entry for each peak/Kovats RI.

## 7. Data analysis

All experimental data were processed using Excel (Office 365) and R (v 3.6.1).

### 7.1 Growth rate estimation

From the OD-curves obtained with the 96-well plates, growth rates (μ) were calculated from the Richards growth model (Candry et al., 2018; F. J. Richards, 1959). After subtracting the average OD of the blanc wells, all OD-values were log-transformed and the Richards equation was fitted using the nls.lm optimization algorithm from the minpack.lm package in R, which yielded the estimation for μ (Elzhov et al., 2016).

## 7.2 Calculations

The calculations on the data from the batch growth, are explained below.

Biomass yields ($Y_X$) were calculated according to Equation 1 with X the microbial biomass expressed as g TSS $L^{-1}$ and C the carbon source as g COD $L^{-1}$, consumed within a time interval $t_2$–$t_1$.

$$Y_X = \frac{X_2 - X_1}{C_2 - C_1} \quad [g\ TSS\ g\ COD^{-1}] \tag{1}$$

Protein yields ($Y_P$) were determined by Equation 2, where P is the protein content in g $L^{-1}$ and C the carbon source as g COD $L^{-1}$, consumed within a time interval $t_2$–$t_1$.

$$Y_P = \frac{P_2 - P_1}{C_2 - C_1} \quad [g\ protein\ g\ COD^{-1}] \tag{2}$$

Biomass productivity ($P_X$) and protein ($P_P$) (g $L^{-1}$ $h^{-1}$) were calculated as the biomass or protein produced (g $L^{-1}$) divided by the growth time (h) (Sui et al., 2019).

These parameters were compared for every strain to assess the impact of the growth phase and the carbon source on the cell's protein content.

## 7.3 Statistical analysis

Statistical analyses were performed to evaluate the effect of the growth phase and carbon source on the protein content, both as the total protein content (mg $L^{-1}$) and as the percentage of TSS. First, the data points of each sampling were categorised manually in to lag, exponential, late exponential, and stationary growth phase. This partition was based on the growth curve obtained by the $OD_{600}$ measurements during the batch growth experiments. A distinction between individual time points was not made within each phase.

The statistical significance of the mean protein content between each growth phase and carbon source was tested via a one-way ANOVA analysis, followed by the Tukey-Kramer post-hoc multiple comparison test. Assumptions were tested via diagnostic plots and formal analysis using the Levene homoscedasticity test (car package) and Shapiro-Wilk's test of normality (Fox, 2019). In case assumptions were not met, the Kruskal-Wallis rank sum test was applied in combination with a Bonferroni corrected Dunn test (dunn.test package), as opposed to ANOVA and Tukey-Kramer (Dinno, 2017; Wuyts, 2004). A significance level of $p < 0.05$ was considered statistically different.

To evaluate the impact of the carbon source and the microbial culture on the aroma profile of the dried product, principal components analyses (PCA) were conducted using the AlphaSoft software (v 12.4, Alpha MOS) of the eNose. PCA graphs were constructed for each strain and for each carbon source. Prior to construction, the peaks from the blanc

were removed from the dataset. Additionally, the peaks with a discrimination power lower than 0.8 were not taken into account, to ensure the most characteristic compounds of each sample were analysed. Since the PCA analyses mainly lead to qualitative conclusions on the similarity of samples, the Euclidian distances between each data cluster were calculated to yield a quantitative comparison of the volatile profiles.

# IV. RESULTS AND DISCUSSION

## 1. Strain identification

The isolate of the formate enrichment was identified as *Paracoccus sp.* while the isolate of the acetate enrichment was *Acinetobacter sp.* The consensus sequences obtained through Sanger sequencing, as explained in section III.5.4, are displayed in Appendix 5.

## 2. Selection of non-limiting carbon source concentration

The growth rate that was derived from the 96-well plate experiments (section III.3), served as the basis to assess the toxicity of acetate and formate. The growth rate (μ, $h^{-1}$) is presented in function of the respective acid concentration (mM) (Figure 5), from which toxicity was evaluated by growth rate limitations. The most suitable carbon source concentrations were then selected accordingly.

Both acids had toxic effects on the growth of *C. necator*, with growth rates decreasing after a dosage of 20 mM acetate and 50 mM formate. *Acinetobacter sp.* did not suffer from toxicity induced by the acetate concentrations applied in this range, however, *Paracoccus sp.* on formate exhibited diminished growth rates at formate concentration from 50 mM. Lastly, the two enriched cultures did not demonstrate a negatively impacted growth by the acid concentrations tested here.

The concentration to be applied during the batch growth was then chosen based on the toxicity results for formate. For formate, a concentration of 50 mM, or 0.81 g COD $L^{-1}$, was selected, which corresponded to an acetate concentration of 12.5 mM to deliver the same COD level.

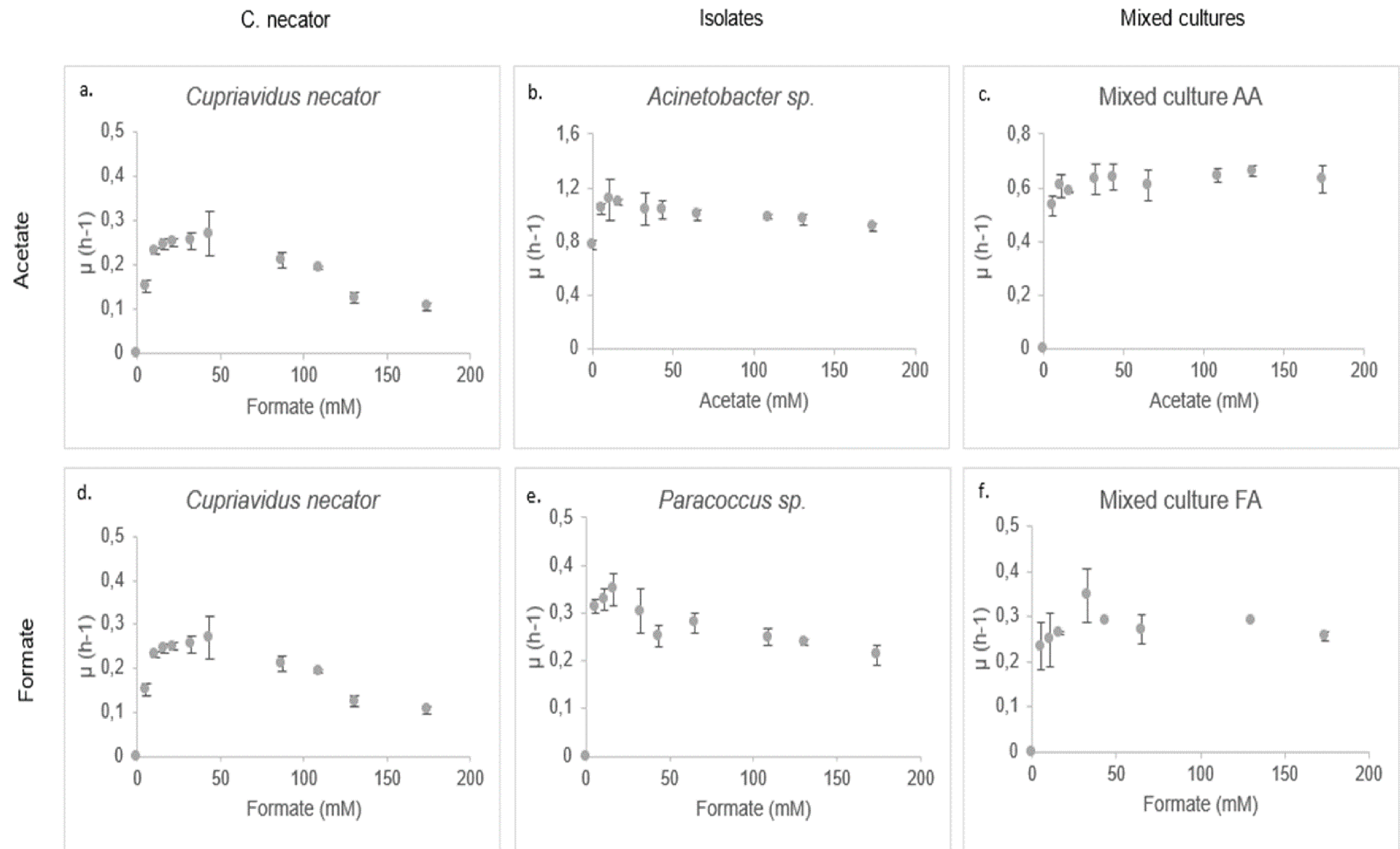



*Figure 5. Growth rate (μ, $h^{-1}$) in function of the applied concentration of acetate or formate (mM) for C. necator, the isolated strains, i.e. Acinetobacter sp. and Paracoccus sp. , and enrichments grown on varying concentrations of acetate and formate in 96-well plates. Abbreviations: AA = acetate, FA = formate*

## 3. Biomass concentration and growth rate in batch growth

The growth rate and biomass concentration reached during the stationary growth phase of the organisms that were included in the batch experiments are presented in Table 4. The procedure for the growth rate calculation was similar to the 96-well plate experiments, as outlined in section 7.1.

*Table 4. Growth rate estimations and stationary phase biomass concentrations for C. necator, the isolated strains, i.e. Paracoccus sp. and Acinetobacter sp., and enrichments grown on acetate and formate in batch growth.*

| Organism | Carbon | Growth rate (μ, $h^{-1}$) | Biomass concentration (mg $L^{-1}$) |
|---|---|---|---|
| *C. necator* | AA | 0.23 ± 0.02 | 518.1 ± 31.1 |
| | FA | 0.14 ± 0.01 | 271.0 ± 21.4 |
| *Acinetobacter sp.* | AA | 0.12 ± 0.01 | 310.0 ± 12.4 |
| *Paracoccus sp.* | FA | 0.04 ± 0.01 | 122.4 ± 1.7 |
| Enrichments | AA | 0.24 ± 0.03 | 487.5 ± 16.5 |
| | FA | 0.05 ± 0.02 | 211.7 ± 14.5 |

The growth rate during the growth on acetate was consistently higher than the growth rate on formate for all organisms included in this study. This can be explained by the more inefficient formate metabolism, compared to the use of acetate as the sole carbon source, as proven in tests conducted with *C. necator* (Yishai et al., 2016).

Comparing the three cultures grown on acetate, the highest values were reached for the enrichment and *C. necator*, while the growth rate of *Acinetobacter sp.* was only half of the other two. For formate, *C. necator* exhibited a much higher growth rate than *Paracoccus sp.* and the enrichment.

Similarly to the growth rates, the biomass concentrations reached were considerably higher for the experiments on acetate than on formate. For all organisms, the biomass concentration was about doubled on acetate compared to formate. These results can be linked to the yields (g g $COD^{-1}$), which are discussed more in depth in section 5. The biomass concentrations, as compared between organisms that were grown on the same carbon source, followed a similar pattern as the growth rates.

# 4. Protein content and concentration

The results for protein, as obtained from the batch growth, were expressed as the protein content of the biomass (%TSS) and as the total concentration (mg $L^{-1}$). The protein contents generally ranged from 30 – 50%TSS, although values up to 80%TSS were observed in *Acinetobacter sp.*, as seen in Figure 6. The influence of the micro-organism, carbon substrate and growth phase on the protein content and concentration is discussed in the next paragraphs.

## 4.1 Influence of the micro-organism on protein content and concentration

As each organism has unique properties, it was expected that the choice of micro-organism would influence the protein content and concentration.

As a preliminary observation, the data across all growth stages was compiled for each organism, to allow for an overall assessment of the influence that the organism had on the protein content (%TSS). In this case, the protein content (Figure 7) was found to be lower in the enrichments than in *C. necator* ($p=0.009$) and the isolates ($p<0.001$). The pure cultures did not exhibit significant differences in protein content, meaning that the enrichments showed a lower overall protein content than all pure cultures in this study.

The results of the total protein concentration (mg $L^{-1}$) (Figure 8), however, lead to an entirely different conclusion. This time, the choice of organism did not have any significant influence on the outcome ($p=0.11$), although this result is likely due to the varying protein contents for each carbon source and in each growth phase (section 4.2 and 4.3), which might level out some differences.

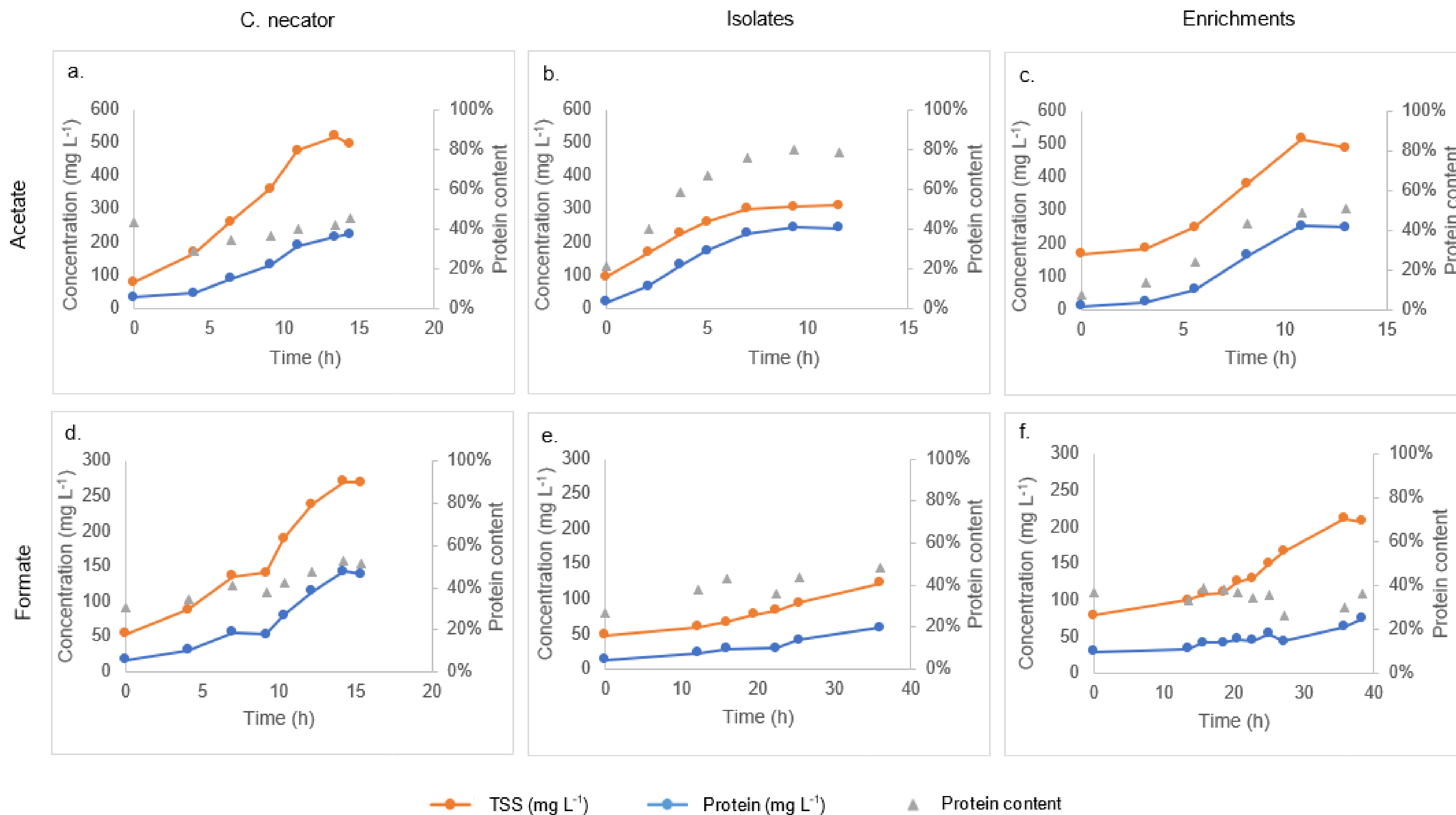



*Figure 6. Biomass concentration, protein concentration, and protein content of C. necator, the isolated strains, i.e. Paracoccus sp. and Acinetobacter sp., and enrichments grown on acetate and formate in batch growth. Biomass and protein concentrations for the growth on acetate ranged to 600 mg $L^{-1}$, and for formate to 300 mg $L^{-1}$.*

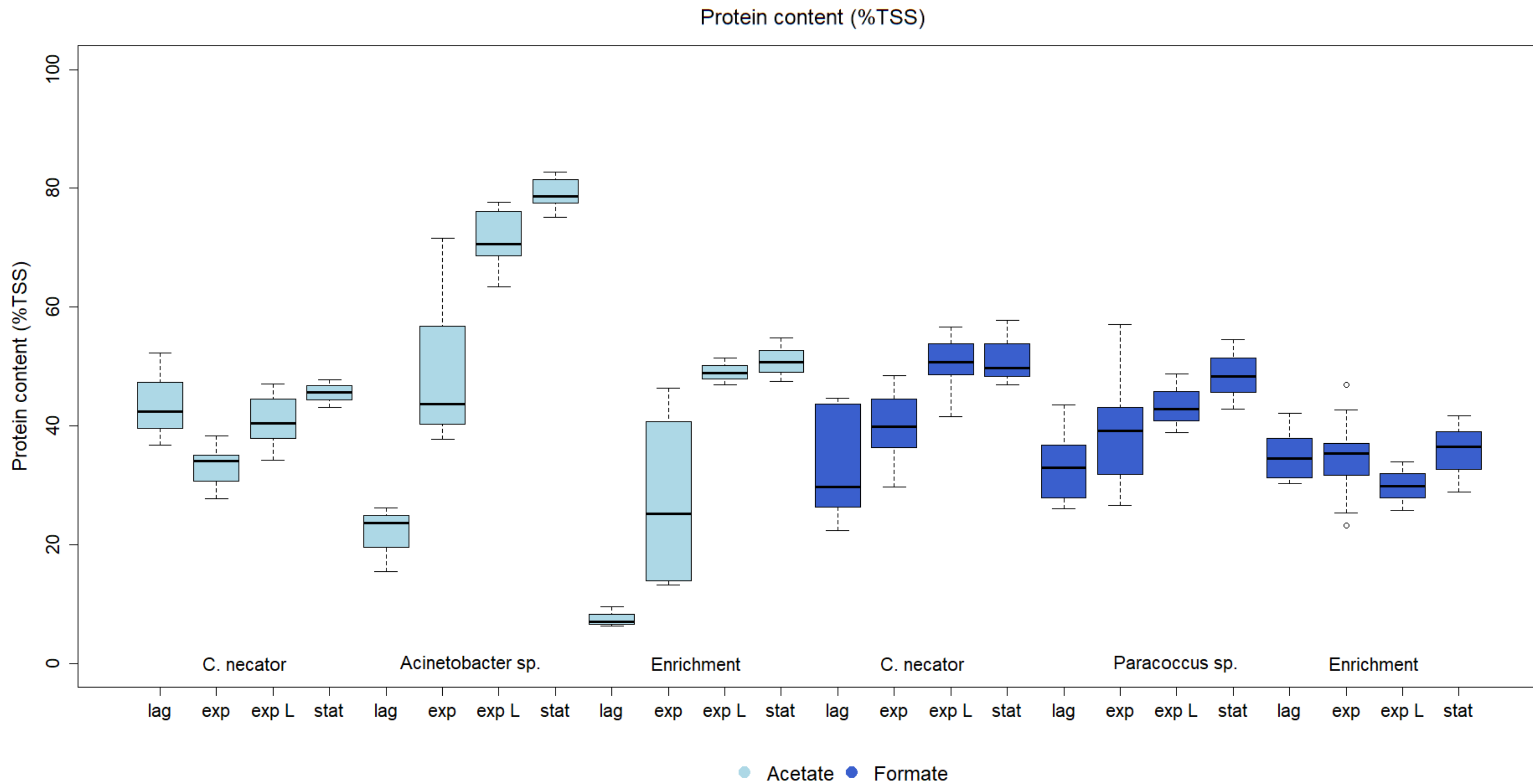



*Figure 7. Protein content (%TSS) in each growth phase of C. necator, the isolates, i.e. Acinetobacter sp. and Paracoccus sp., and enrichments grown on acetate and formate in batch growth.*

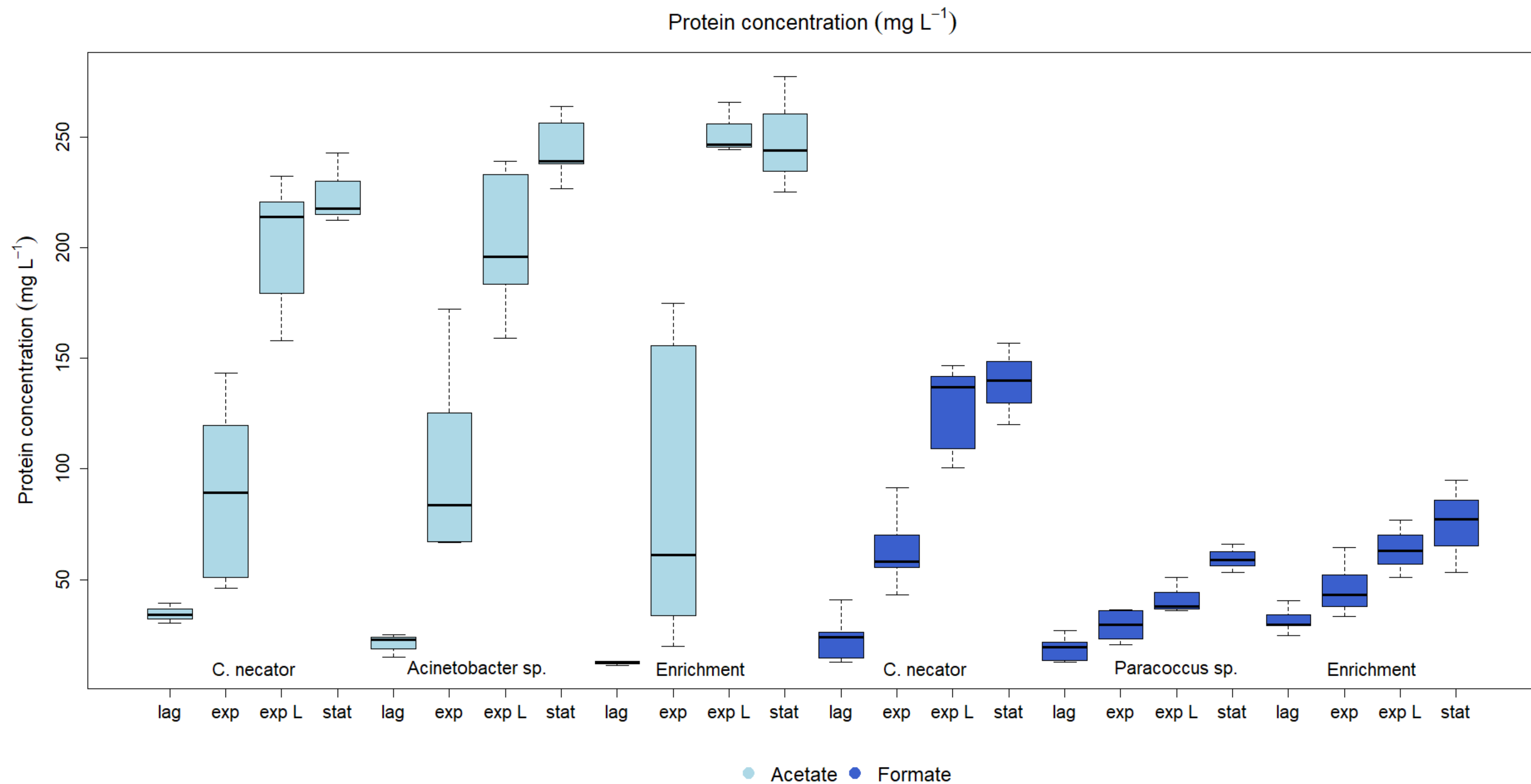



*Figure 8. Protein concentration (mg L$^{-1}$) in each growth phase of C. necator, the isolates, i.e. Acinetobacter sp. and Paracoccus sp., and enrichments grown on acetate and formate in batch growth.*

## 4.2 Influence of the carbon source on protein content and concentration

The carbon substrate that the organisms were supplied with could also impact the growth and protein production. The effect of acetate and formate was evaluated for each species because of the previously established influence of the organism on protein levels.

The protein content was first evaluated as the average across all growth phases. The protein content of *C. necator* was found to be significantly higher when grown on formate ($p=0.02$), yet the isolates exhibited greater protein contents on acetate ($p<0.001$). The enrichments on the other hand, showed no statistical influence of the carbon source on their average protein content. It should be noted though that the two isolates and the two enrichment cultures technically cannot be compared side by side as the compositions were not the same in the acetate and formate cultures. As *C. necator* was the only strain present in both carbon substrate batch tests, this experiment did allow for a direct comparison of the protein content to assess the influence of the carbon source.

For this reason, the differences in microbial composition should be taken into account as well. As the choice of micro-organism also influences the protein content, as previously stated, the varying protein content could not be solely contributed to the change in carbon substrate, especially since this effect was not very distinct in the *C. necator* experiments. From these results, it could be concluded that the micro-organism is the most influential factor on the protein content, rather than the carbon that was supplied to the organism.

Next, the data for the total protein concentration (mg $L^{-1}$) were also compared for each organism and carbon source. For both *C. necator* and the isolates, the total concentration was higher on an acetate regime ($p<0.001$). This result was to be expected for the isolates, as it was already found that they exhibited higher protein content (%TSS) and higher biomass concentration during the acetate based growth. Yet, for *C. necator* this conclusion is not as straightforward. Given that the protein content was statistically higher with formate as a carbon source, the biomass concentration had to be considerably increased to attain a higher total protein concentration during the acetate based growth. Indeed, the TSS values of the acetate batch test were about doubled compared to formate, although an equivalent increase was observed during the tests with the other organisms. This biomass increase could thus not explain the higher protein concentration during the acetate growth. This could be explained by the absolute values of the protein content for each carbon source, which were very similar, even though statistically higher for formate, as mentioned earlier. The minimal differences in protein content were thus negated by the much larger

increase in biomass content to result in a higher overall protein concentration in the acetate grown culture.

The enrichments showed no significant difference of protein concentration between the two carbon substrates. This result is quite counterintuitive, given that the protein content was not significantly different and the biomass concentration was considerably higher during the acetate growth. The total protein concentration was thus expected to be greater in the acetate supported culture. This contradictory result could be explained by the much wider range in protein concentration in the acetate culture (12 – 249 mg $L^{-1}$) than in the formate culture (29 – 75 mg $L^{-1}$). Even though not statistically different as a whole, the final protein concentration was indeed higher during the batch experiment with acetate as the carbon source, as assumed.

The counterintuitive results of both the protein content and concentration suggest the need to look into the comparisons of each growth phase for every organism and carbon source, rather than treating the data as a whole.

## 4.3 Influence of the growth phase and growth rate on protein content and concentration

As microbial growth displays distinctive phases, these might also alter the protein content of the cells. Contrarily to the total concentration of protein, which rises as the biomass increases throughout the growth, the effect of the growth phase on the protein content of the cell cannot be readily deducted.

Because of the already observed influence of the micro-organism, as well as the carbon source that the organism was grown on, the influence of the growth phase was assessed for each strain and carbon substrate. The statistical results of this analysis are summarised in Table 5. In case a growth phase is not mentioned, no significant effects were observed.

*Table 5. Statistical results of the influence of the growth phase on protein content (%TSS) and concentration (mg $L^{-1}$). Abbreviations: AA = acetate, FA = formate, exp = exponential, expL = late exponential, stat = stationary phase.*

| Organism | Carbon | Protein content (%TSS) | Protein concentration (mg $L^{-1}$) |
|---|---|---|---|
| *C. necator* | AA | exp <* (lag=expL=stat) | (lag=exp) <*** (expL=stat) |
| | FA | lag <** (expL=stat) | lag <*** exp < ***(expL=stat) |
| *Acinetobacter sp.* | AA | lag <*** exp <*** (expL=stat) | lag <** exp <*** (expL=stat) |
| *Paracoccus sp.* | FA | No significant effect | (lag=exp) <* stat |
| Enrichments | AA | lag <* (expL=stat) | (lag=exp) <*** (expL=stat) |
| | FA | No significant effect | lag <* exp <* (expL=stat) |

Significance level

| | | | |
|---|---|---|---|
| * | p < 0.05 | < | Significantly smaller |
| ** | p < 0.01 | = | No significant difference |
| *** | p<0.001 | | |

As illustrated in Figure 7, a clear trend of increasing protein contents with progressing growth was observed, which was also confirmed by the statistical analysis (Table 5). The protein content within each cell differed the most when transitioning from the lag/exponential growth to the late exponential/stationary phase.

Though, for the formate supplemented growth of *Paracoccus sp.* and the enrichment no significant influence of the growth phase on the protein content was found. For *Paracoccus sp.*, a rise in protein content is still noticeable in the graph, even though not statistically significant. However, the formate enrichment displayed a rather constant protein content with no sign of an increase throughout the growth. Additionally, for *C. necator* on acetate, the protein content of the cell was lower during the exponential phase than during any other phase.

Originally, it was hypothesised that the protein content would be the highest during the exponential phase, when the highest growth rates were reached and thus the greatest abundancy of protein-producing ribosomes (Bremer et al., 2008; Klumpp et al., 2009; Volova et al., 2010). Contrarily to this assumption, the protein content did not exhibit a peak value during the exponential phase, but either continued to increase to the end of the growth cycle, or did not seem to be impacted by the change in growth phase.

This variation was not as distinct in *C. necator* given that the average protein levels at each timepoint ranged from 29 to 46%TSS for acetate and from 30 to 53%TSS for formate.

For the isolates on the other hand, this effect was the most pronounced with average protein contents varying from 22%TSS up to 80%TSS during the acetate based growth of *Acinetobacter sp.*, while *Paracoccus sp.* exhibited a substantially more narrow protein range from 27 to 49%TSS. The enrichments also followed this trend of a greater variation in protein contents when supplied with acetate, with protein making up between 7 and 51%TSS of the biomass on average versus a more constant protein content between 26 and 38%TSS during the formate experiment.

Even though this trend might not seem to correlate to the initial hypothesis, the protein content did, however, increase more rapidly during the exponential growth phase in case of the isolates and the enrichment grown on acetate (Figure 6b,c), resulting in a sigmoid curve. The lag and stationary phase exhibited a more gradual protein gain, which could be attributed to the lower growth rates and thus lower ribosome activity (Clark et al., 2013). These observations gave the impression that the growth rate, and thus growth phase, did play a role in these protein content results, albeit not as initially assumed.

The main issue with the initial hypothesis was that the storage of the already produced protein had not been taken into account yet. The protein content was only thought of as the protein produced at that specific timepoint, without considering the history of the cell's protein synthesis. If protein is stored in the cell, e.g. as a building block or as an organelle, than it is not the total protein content that is dependent of the growth rate, but rather the protein production rate (Cole, 2016; Hintsche et al., 2013). Evidently, if the protein is stored in the cell, it will accumulate over time, thus explaining the ever-increasing protein content and the sigmoid curve (Figure 6b,c).

This sigmoid profile of the protein content was, however, not observed in all batch experiments. The enrichment culture grown on formate exhibited a more constant course, for example. This might be because of the multiple organisms present, which could all be dominant at different times. It could be the case that each individual organism did exhibit this sigmoid profile, but that the combined protein content did not experience this, because of the shifted expression of each organism.

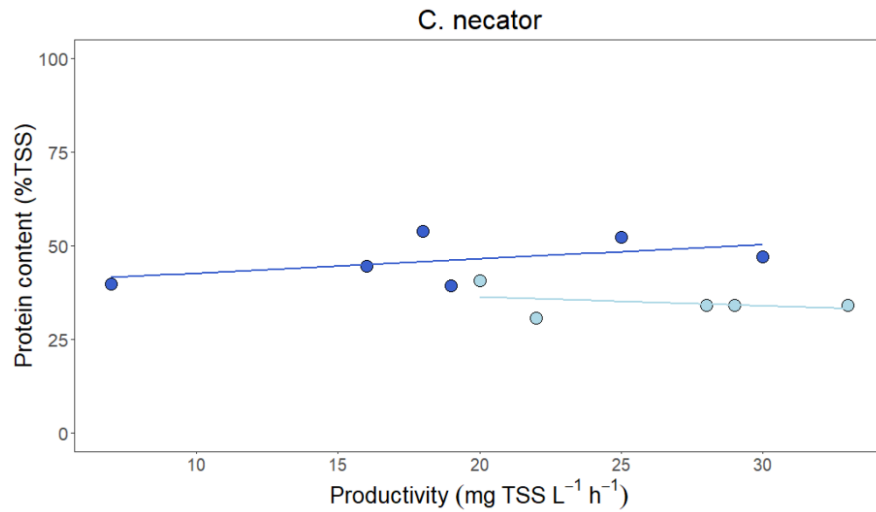
From this point of view, one might consider the growth rate as being a more influential factor on the protein content, rather than the actual growth phase (Klumpp et al., 2009). To this end, the protein content of each organism was assessed as a function of the biomass productivity (mg TSS $L^{-1}$ $h^{-1}$), which was employed as a measure for the growth rate (Figure 9). The productivity was chosen for this parameter, rather than the growth rate, as this approach allowed following this parameter through time. Some of the obtained regression curves did portray a positive correlation, with higher protein contents for higher

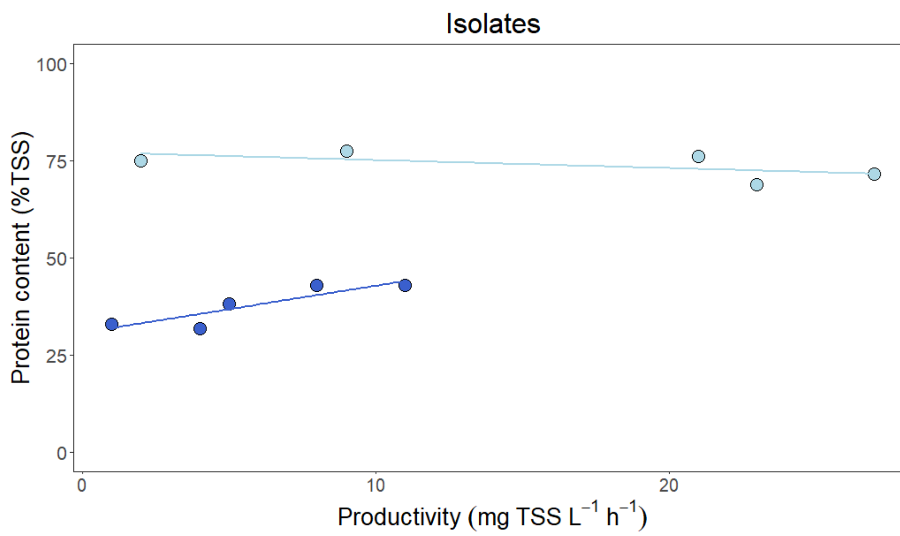
productivities, which was in line with the theoretical expectations. However, the acetate isolate *Acinetobacter sp.* and *C. necator* grown on acetate exhibited a negative correlation. This could be explained by the opposing effect of the increased protein dilution at higher growth rates. As the bacterial growth progresses, the cell volume continues to expand, causing the amount of protein per unit biomass to decrease (Hintsche et al., 2013; Klumpp et al., 2009). This effect can be especially influential at faster growth, causing the overall protein content, when expressed as percentage of the biomass, to decrease (Klumpp et al., 2009). A third growth dependent process that could affect the protein content, would be the protein degradation, although this phenomenon is not likely to have a large impact unless at slower growth (Hintsche et al., 2013).

A further observation from Figure 9 was that the growth of the formate fo seemingly lacked a correlation between the protein content and the biomass productivity. Even though it seems that the protein content is not affected by the growth, it is more likely that the opposing effects of protein synthesis, degradation, and dilution result in a dynamic equilibrium.

These differing regression curves also illustrate the varying growth rate dependency of each protein (Pedersen et al., 1978). To this end, the expression of a whole proteome and thus the protein content in function of the growth rate cannot be surely predicted (Klumpp et al., 2009).

Earlier, the influence of the carbon source on protein content was assessed (section 4.2), However, it could be the case that the specific carbon source is not responsible for eventual differences in protein content between the two, but rather the difference in exponential growth rate induced by the two carbon substrates (Klumpp et al., 2009). On the other hand, a research on *E. coli* revealed that the ratio of growth rate dependent proteins to the total amount of proteins differs based on the medium composition and thus carbon source (Pedersen et al., 1978).



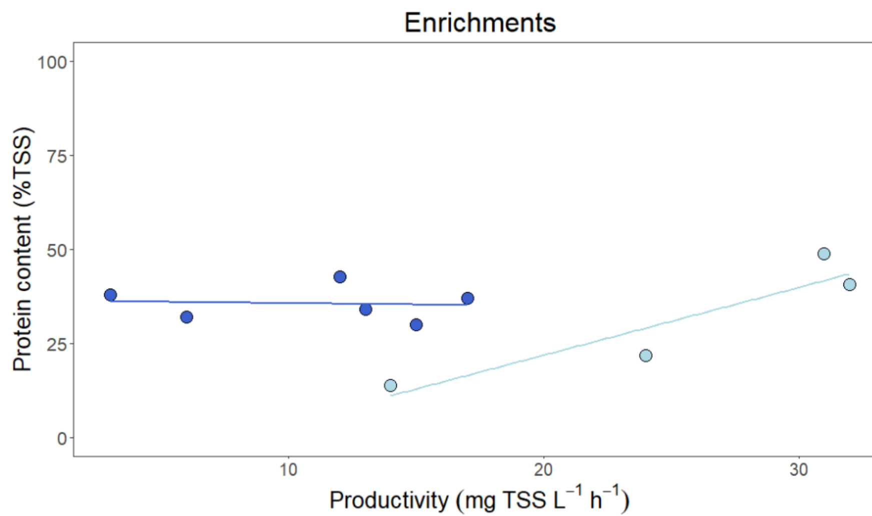




*Figure 9. Protein content (%TSS) in function of the biomass productivity (mg TSS $L^{-1}$ $h^{-1}$) for C. necator, the isolated strains, i.e. Acinetobacter sp. and Paracoccus sp., and enrichments grown on acetate and formate in batch growth.*

# 5. Yields and productivity

Next to assessing the protein content and concentration, the protein productivity and yield were calculated to gain more in depth knowledge of the change in protein throughout the growth. The same characteristics were also determined for the cell dry weight.

## 5.1 Influence of the micro-organism and the carbon source on the protein and biomass yields and productivity

Since the micro-organism and choice of carbon substrate affected the protein content and concentration, these factors might be equally influential on the yields and productivity. The average values, across all growth phases, for these characteristics are presented in Table 6.

*Table 6. Average biomass and protein yields and productivities for C. necator, the isolated strains, i.e. Acinetobacter sp. and Paracoccus sp., and enrichments grown on acetate and formate in batch growth. Abbreviations: AA = acetate, FA = formate, TSS = total suspended solids, COD = chemical oxygen demand*

| Organism | Carbon | Yield (g g COD$^{-1}$) | | Productivity (mg L$^{-1}$ h$^{-1}$) | |
|---|---|---|---|---|---|
| | | TSS | Protein | TSS | Protein |
| C. necator | AA | 0.48 | 0.17 | 29.84 | 10.60 |
| | FA | 0.27 | 0.13 | 12.44 | 6.29 |
| *Acinetobacter sp.* | AA | 0.36 | 0.33 | 28.90 | 25.99 |
| *Paracoccus sp.* | FA | 0.11 | 0.07 | 1.49 | 0.99 |
| Enrichments | AA | 0.35 | 0.24 | 20.63 | 14.32 |
| | FA | 0.25 | 0.07 | 2.69 | 0.77 |

All parameters assessed here exhibited higher values for the organisms grown on acetate compared to formate. The same amount of feedstock consumption, expressed as COD, thus resulted in more biomass and protein, when it is in the form of acetic acid instead of formic acid. Additionally, the time and working volume needed to produce this amount of biomass and protein was lower with acetate as the carbon source because of the higher productivities.

Especially the drastically reduced productivities for the formate-supported growth of the isolates and the enrichments are notable compared to the values found during the experiment with acetate. Of course, as these cultures were not the same, this comparison is not completely justified. However, the productivities on formate are notably lower than the yields, when assessed as the ratio with the acetate values. For example, the protein yield of the isolate on formate is 4.4 times lower than on acetate, whereas the productivities are reduced with a factor 26. These reduced productivities can be explained by the much

longer growth duration of these 2 formate cultures, which is also reflected in the reduced growth rates on formate (section 3). The average yield is not affected by this duration, as the amount of supplied feedstock was equal in every batch test.

Taking a closer look at the yields of the acetate based growth, it is clear that *C. necator* exhibited the highest biomass yield. However, for protein, this organism exhibited the lowest yield, which suggests a trade-off between the production of either more protein or more biomass with the supplied nutrients. Still, this trend was not really observed in the results from the other cultures. This observation of a trade-off is discussed further in section 5.3.

The protein to biomass yield ratios differed depending on the organism, confirming a strain-specific level of protein production, as discussed previously. *C. necator* yielded 2.9 times more biomass per g COD than that it produced protein, whereas the two yields from the isolated strains were quite similar with only 1.1 times more biomass than protein. The enrichments' results were intermediate to the two other experiments, with a biomass/protein ratio of about 1.5. The general order of these results can also be obtained from the curves of the protein and biomass concentration. The increasing distance between the protein and biomass curve for *C. necator* (Figure 6a) reflects the large ratio of the yields, while the curves obtained by *Acinetobacter sp.* (Figure 6b) and the acetate enrichment (Figure 6c) follow a rather parallel path.

No clear trend was observed in the yields when comparing the enrichments with their respective isolate. For acetate, the yields of *Acinetobacter sp.* were higher for both protein and biomass, whereas this was the opposite for *Paracoccus sp.* and the formate enrichment.

Next, the productivity indicated the time and working volume needed to produce a certain amount of biomass or protein. Biomass productivity for the acetate-based cultures was the highest during the growth of *C. necator*, just as the yield was the highest for this organism. Nevertheless, this strain produced protein at a rate almost 3 times smaller, the same biomass/protein ratio that was observed for the yields. All three strains exhibited comparable biomass/protein productivity ratios as their respective yield ratios, indicating a clear correlation between the yield and productivity. Productivity of the isolates' biomass was almost as high as *C. necator*, but with a much higher protein productivity.

Analysis of the growth on formate revealed that both the highest biomass and protein yields were reached during the batch growth of *C. necator.* Similarly to the acetate yields, the ratios of protein/biomass depended on the organism, although these ratios differed from those of the acetate facilitated growth. *C. necator* yielded half the amount of protein

as the amount of biomass when consuming the same amount of COD, and the isolates produced about 1.5 times more biomass than protein. The enrichments biomass yield was as much as 3.4 times higher than its protein yield, which is also illustrated by the increasing distance between the biomass and protein concentration curves (Figure 6f). Comparable values were observed for the productivity ratios, as was the case for the growth on acetate.

## 5.2 Influence of the growth phase on the protein and biomass yields and productivity

To assess how these characteristics evolved throughout the organisms' growth, the yields and productivities are presented in function of time (Figure 10). Firstly, it can be noted that the yields follow a more trend, while the productivities tend to change more throughout the growth.

For the biomass, maximum yields and productivities were reached during the exponential/late exponential phase, which was to be expected since a microbial culture grows the fastest during the exponential growth phase. After this phase, additional feedstock consumption resulted in comparable or lower yields, and decreasing productivity. Further growth lead to decreasing productivities, which is very pronounced during the growth of *Acinetobacter sp.* (Figure 10).

For protein, the yields exhibited a similar trend as for the biomass with the maximum yields reached during the exponential/late exponential phase. Protein productivities also increased to their highest values during the exponential/late exponential growth phase. As the protein productivity increased to its maximum during this phase, protein was produced at the highest rate, rapidly increasing the cell's protein content. This result gives a clear indication of the influence of the growth phase, or rather growth rate, on the protein content, as discussed in section 4.3.

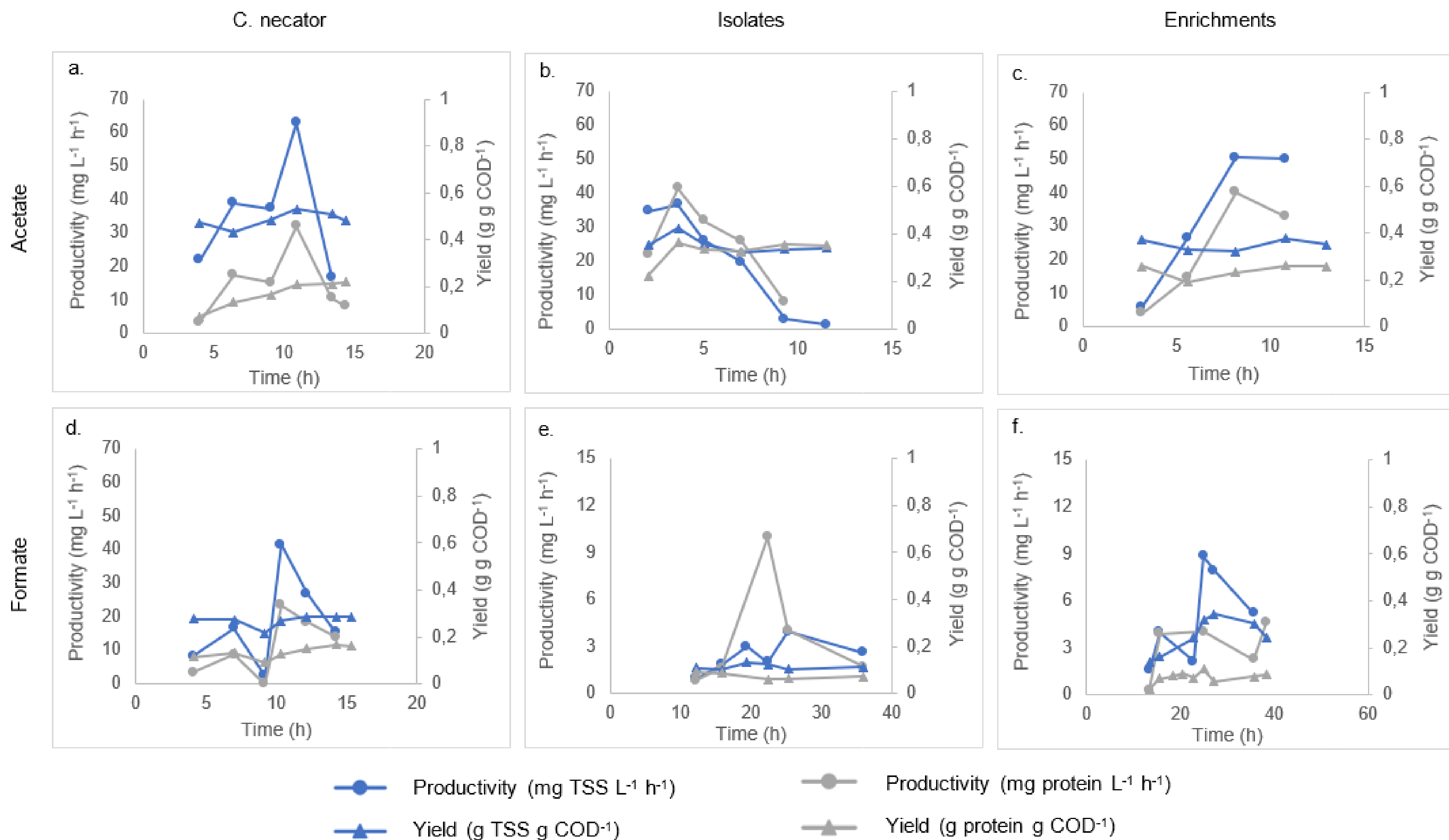



*Figure 10. Productivities (●) and yields (▲) for biomass (blue) and protein (grey) production for C. necator, the isolates, i.e. Acinetobacter sp. and Paracoccus sp., and enrichments grown on acetate and formate in batch growth. Productivities for the growth on acetate ranged to 70 mg $L^{-1}$ $h^{-1}$, as well as for C. necator on formate. The range for the productivities of Paracoccus sp. and the formate enrichment was lower, up to 15 mg $L^{-1}$ $h^{-1}$.*

## 5.3 Influence of the protein content on the biomass productivity

The influence of the productivity on the protein content had already been assessed, however the protein content can also influence the exponential growth rate (Goelzer et al., 2011; Terradot et al., 2018). As protein production also requires nutrients, bacteria are forced to re-allocate their available resources, leading to a lower amount available for the biomass production, so that a trade-off between biomass and protein has to be made (Terradot et al., 2018). This phenomenon of a reduced growth rate due to protein production is called the protein cost or protein burden (Goelzer et al., 2011).

However, as in this study multiple organisms and substrates were used, that also influence the biomass growth rate, this effect could not be properly assessed and is thus not further discussed.

# 6. Aroma profile

## 6.1 The influence of the micro-organism and carbon source on the aroma profile

The obtained aroma profiles were processed by use of multivariate statistics, more specifically principal components analyses (PCA). A PCA analysis performs an orthogonal transformation on the dataset to yield a reduced set of independent orthogonal variables, called the principal components (PC). This reduction allows for a simplified graphical representation of the dataset to assess the similarity of each sample (Wardencki et al., 2013).

To obtain a first glance at the data, all samples were included, of which the result is presented in Figure 11. In this case, a 2-dimensional system was chosen for ease of representation, with PC1 and PC2 the components that account for the highest and second highest variability, respectively. PC1 thus accounts for 37.09% of the variability between the samples, and PC2 for 27.77% of variability. Given that these percentages only amount up to a combined 64.86%, about 35% of the variability between the samples is not displayed nor taken into account in this graph. Because of this low component load, PCA did not allow to fully discriminate these samples based on the carbon source or the organism and conclusions from this graph should thus be handled cautiously (Rottiers et al., 2018). As not all variance is explained by the two components, it is quite possible that

samples that seem close to each other are in reality further apart because of an extra PC that accounts for their differences.

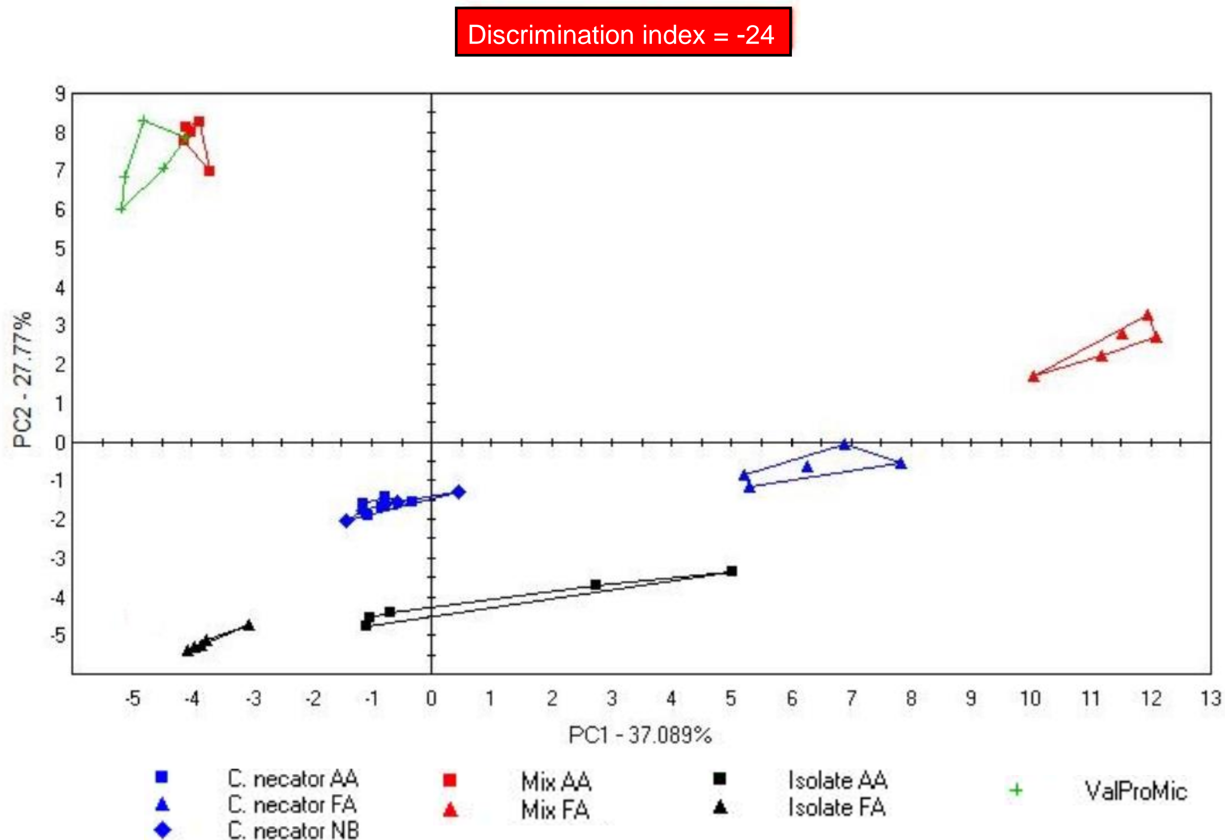



*Figure 11. PCA analysis of the volatile profiles of all samples. Abbreviations: AA = acetate, FA = formate, Mix = enrichment, isolate AA = Acinetobacter sp., isolate FA = Paracoccus sp.*

Each point in the graph represents one of the 5 replicates that were analysed for every sample. The lines joining the samples show the clustered data. As two samples of the acetate isolate *Acinetobacter sp.* were quite far from the rest, these were considered outliers and were not taken into account in further analyses.

First off, it was clearly visible that the volatile profiles of most samples could be distinguished from any other sample, as there was little overlap of the clusters. However, this observation was not confirmed by the negative discrimination index of -24. A positive discrimination index indicates that the samples' volatile profiles are significantly different, with increasing difference as the index increases up to 100 (Zhu et al., 2004). Though this negative value can be attributed to the minimal differences between the samples of *C. necator* grown on acetate and nutrient broth. When the nutrient broth sample was not included in the PCA analysis, the discrimination index rose up to +84, exhibiting a definite variation between the samples' volatile profiles. From this rather simple observation, a much more important conclusion can be drawn: the eNose can be effectively used as a means to discriminate between the aroma of each sample.

The similarity of the samples can also be assessed qualitatively from this PCA analysis, where clusters that are further apart account for greater differences. To further distinguish each sample, quantitative measurements were provided by the Euclidian distances between each data cluster. Just like in the graph, a greater distance accounted for more diverse volatile profiles. These results are summarised in Table 7.

*Table 7. Euclidian distances between dried samples of C. necator, the isolates, and the enrichments grown on acetate and formate in batch growth, and ValProMic. Abbreviations: Cnec= C. necator, Isol AA= Acinetobacter sp., Isol FA= Paracoccus sp., Mix= enrichment, AA= acetate, FA=formate, NB= nutrient broth.*

| Strains | Cnec AA | Cnec FA | Cnec NB | Isol AA | Isol FA | Mix AA | Mix FA |
|---|---|---|---|---|---|---|---|
| Cnec FA | 9.07 | x | x | x | x | x | x |
| Cnec NB | 5.25 | 8.32 | x | x | x | x | x |
| Isol AA | 7.82 | 8.51 | 7.70 | x | x | x | x |
| Isol FA | 7.51 | 12.47 | 7.42 | 6.70 | x | x | x |
| Mix AA | 13.37 | 16.21 | 13.95 | 15.33 | 14.81 | x | x |
| Mix FA | 14.08 | 9.47 | 14.45 | 13.71 | 17.21 | 17.9 | x |
| Valpromic | 13.14 | 15.6 | 12.43 | 15.29 | 15.01 | 14.99 | 19.03 |

| Legend | <10 | >10 | >15 |
|---|---|---|---|

The influence of the organism on the volatile profile was assessed first. As the organisms that were grown on the same carbon source, either acetate or formate, were quite scattered in the PCA graph, they did not display any evident relations at first sight. For acetate, the aromas from *C. necator* and *Acinetobacter sp.* were the most similar (7.82), with the enriched culture being further apart from both *C. necator* (13.37) and *Acinetobacter sp.* (15.33). For formate on the other hand, *C. necator* and the enrichment were closer (9.47) and *Paracoccus sp.* had a more differing pattern with a distance of 12.47 to *C. necator* and 17.21 to the formate enrichment. Quite interestingly, both of the isolates exhibited a volatile profile with a low similarity to their respective enriched culture. This could indicate that the isolated strains were not very dominant in the microbial composition of the enrichments.

Evaluating the influence of the carbon source, it seems that the two enrichments had the most differing VOC's out of the three pairs. With the two data clusters on opposite sides of the graph and a distance of 17.90, the second highest value, this should account for

distinct aroma profiles. This large difference can likely be attributed to their versatile composition due to the enrichment procedure. Through multiple stages of inoculation of fresh medium and addition of the respective carbon sources, the composition of the cultures changed slowly but surely, yielding two very diverse microbiomes, each accounting for their own set of VOC's. Thus, not only the carbon source varied between these samples, but also the microbial composition, leading to two vary distinct aroma profiles.

The volatile profiles of the isolates and *C. necator* seemed to be less impacted by a change in carbon source, with considerably smaller differences between the data clusters. Still, the clusters could be easily distinguished in the graph, indicating definite variation, which is also reflected in the distances. What was quite striking, is that the distance between the two isolates, *Acinetobacter sp*. and *Paracoccus sp*., was smaller than the distance between the acetate and formate samples for *C. necator*, both in Figure 11 and in Table 7. Since both the microbial culture and the carbon source varied for the isolates, one would expect that these would differ more than for *C. necator*, which is thus not the case. Quite the opposite actually, as the distance between the two isolates was found to be the second smallest out of all comparisons. As *C. necator* can rapidly adapt to carbon source variations and as growth conditions have been shown to influence volatiles, it is likely that *C. necator* produced different metabolites because of the altered metabolism resulting in differing VOC emissions(Choudhary et al., 2017; Lu et al., 2016). This could explain the difference between the two aroma profiles. The sample grown on nutrient broth showed a smaller difference to the acetate sample than to the formate sample, which could indicate that the use of formate had a more influential impact on the volatile profile than the use of acetate.

The influence of the carbon source on the variety in volatile profile was in general smaller than the influence of the micro-organism. Yet, as *C. necator* is the only true comparison for the influence of the carbon source on the same organism, further testing would be required. Between both carbon sources, *C. necator* displayed a Euclidian distance of 9.07, thus on the lower side, whereas values for a differing organism went up to 17.21 between the formate enrichment and *Paracoccus sp*. Comparing C. necator to a different organism that was grown on the same carbon source, then the largest difference was found between the acetate enrichment and *C. necator* on acetate with a distance of 13.37. Still, the influence of the carbon source versus the micro-organism was not consistent in these results. It is thus possible that not the organism or the carbon source impacted the volatile profile, but rather the metabolism that is applied by the organism, since a metabolism shift

will likely impact the metabolites that are produced. This way, the volatile profile can also be affected.

Apart from the influence of the organism and carbon source among the samples from this study, these samples were also compared to the industrial ValProMic. This was quite interesting, as all comparisons exhibited high distances. As was the case with the enrichments, these samples did not only contain a diverse microbial culture, but were also grown on an entirely different growth medium. ValProMic was cultivated with potato waste streams as feedstock, which is mainly composed of water, starch and other carbohydrates, and fibres (Ahokas et al., 2014; Avecom, 2016). This medium composition is by no means comparable to the mineral salts medium with acetate or formate as a carbon source. Additionally, the downstream processing, such as the drying process, is likely to differ considerably from the procedure applied for the samples in this study, which might account for further differences in the volatiles. These varying conditions could thus explain the large variety between the ValProMic samples and the ones obtained during this research. The process applied for ValProMic was not known. In fact, the greatest distance and thus difference in aroma, was found to be between the ValProMic sample and the enrichment grown on formate.

What is also quite striking is the seemingly opposite conclusion that could be drawn from the distance between ValProMic and the acetate enrichment in Table 7, versus in the PCA graph (Figure 11). The graph shows the data clusters close to each other, whereas the Euclidian distance of 14.99 suggests a more differing aroma profile. This could be clarified by the low component loading of the two PC's from the PCA leading to a loss of variance that is accounted for in the graph, as explained before.

## 6.2 Identification of volatile compounds and odour descriptors

In order to be able to describe the differences between each samples' aroma, the volatile compounds were identified through AroChemBase, as described in section III.6.4. Based on the Kovats indices, a potential component was then identified for each peak, or sensor. It should be mentioned though that identification by Kovats RI based on retention times only yields tentative results. As the eNose is more suitable for exploratory analyses, other analytical techniques, such as mass spectrometry, are more appropriate to obtain detailed information about the volatile compounds (Rottiers et al., 2018). It can thus not be guaranteed that the compounds identified here are the actual volatiles present in the sample.

*Table 8. Volatile compounds identified by the eNose in dried samples of C. necator, the isolates, i.e. Acinetobacter sp. and Paracoccus sp., and enrichments grown on acetate and formate in batch growth. Average peak areas of 5 replicates per sample are displayed as mean ± standard deviation. Abbreviations: AA=acetate, FA=formate, NB=nutrient broth.*

| Kovats RI | Volatile compounds | Odour descriptor [a] | **Average peak area (mean ± standard deviation)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *C. necator* AA | *C. necator* FA | *C. necator* NB | *Acinetobacter sp.* | *Paracoccus sp.* | Enrichment AA | Enrichment FA | ValProMic |
| | **MXT-5 column** | | | | | | | | | |
| 656 | Trichloroethane | Sweet | 1306 ± 364 | 2477 ± 230 | 2434 ± 798 | 57 ± 0 | - | 12074 ± 904 | 11100 ± 865 | 9083 ± 382 |
| 666 | Trichloroethane | Sweet | 521 ± 195 | 1616 ± 163 | 1490 ± 897 | - | - | 11883 ± 1050 | 9018 ± 1028 | 7671 ± 442 |
| 680 | Pentan-2-ol | Fermented | 339 ± 278 | 284 ± 312 | 162 ± 124 | 56 ± 0 | 123 ± 95 | 4783 ± 973 | 1387 ± 382 | 6394 ± 267 |
| 758 | (E)-2-penten-1-ol | Earthy | 1052 ± 33 | 895 ± 146 | 875 ± 109 | - | - | 639 ± 142 | 359 ± 131 | 2496 ± 211 |
| 803 | Ethyl butyrate | Fruity | 496 ± 46 | 578 ± 89 | 553 ± 87 | 56 ± 0 | 103 ± 19 | 660 ± 111 | 1069 ± 75 | 2239 ± 200 |
| 895 | Cyclohexanone | Minty | 20 ± 1 | 63 ± 11 | 70 ± 12 | - | - | 119 ± 7 | 84 ± 9 | 515 ± 44 |
| 906 | Heptan-2-ol | Fruity | 354 ± 25 | 507 ± 34 | 427 ± 21 | 126 ± 9 | 141 ± 14 | 282 ± 17 | 471 ± 22 | 768 ± 83 |
| 923 | α-pinene | Pine | 1102 ± 22 | 1229 ± 41 | 829 ± 36 | - | - | 2134 ± 70 | 862 ± 24 | 2132 ± 96 |
| 1 058 | Benzyl alcohol | Fruity | 2476 ± 72 | 10096 ± 1263 | 3226 ± 231 | 8120 ± 234 | 1777 ± 164 | 1900 ± 123 | 9182 ± 470 | 679 ± 501 |
| 1 215 | 6-decenal | Green | 2498 ± 87 | 5261 ± 429 | 2857 ± 99 | 3368 ± 233 | 1595 ± 96 | 938 ± 20 | 6590 ± 238 | 285 ± 61 |
| 1 283 | Anisyl alcohol | Floral | 6078 ± 75 | 5842 ± 75 | 5928 ± 262 | 6865 ± 834 | 4124 ± 221 | 2639 ± 384 | 23121 ± 1060 | 1253 ± 161 |
| 1 466 | Geosmin | Earthy | 611 ± 66 | 1378 ± 51 | 674 ± 123 | 309 ± 44 | 294 ± 72 | 230 ± 4 | 2698 ± 98 | 151 ± 16 |
| 1 548 | Rheosmin | Fruity | 307 ± 61 | 549 ± 7 | 230 ± 75 | 64 ± 13 | 63 ± 14 | 51 ± 4 | 886 ± 34 | - |
| 1 726 | Methyl tetradecanoate | Floral | 323 ± 30 | 190 ± 28 | 197 ± 13 | 94 ± 15 | 105 ± 15 | 2547 ± 175 | 924 ± 62 | 131 ± 16 |
| 1 773 | Octyl caprylate | Fruity | 92 ± 9 | 70 ± 11 | 68 ± 10 | 63 ± 0 | 63 ± 5 | 1194 ± 61 | 153 ± 57 | 80 ± 4 |
| | **MXT-1701 column** | | | | | | | | | |
| 663 | 1-propanethiol | Cabbage | 1562 ± 192 | 3836 ± 589 | 4338 ± 751 | - | - | 21788 ± 1938 | 19701 ± 2009 | 10815 ± 1107 |
| 1 060 | Dimethyl Sulfoxide | Cabbage | 2250 ± 186 | 10215 ± 1493 | 3094 ± 207 | 8400 ± 247 | 1855 ± 176 | 1733 ± 154 | 9359 ± 515 | 1529 ± 219 |
| 1 105 | Ethyl (methylthio)acetate | Fruity | 2591 ± 344 | 5109 ± 503 | 2414 ± 308 | 3210 ± 171 | 835 ± 138 | 2620 ± 162 | 5721 ± 366 | 3735 ± 208 |
| 1 278 | 6-decenal | Green | 1274 ± 186 | 4245 ± 384 | 1929 ± 225 | 1803 ± 93 | 1154 ± 117 | 520 ± 88 | 7664 ± 439 | 68 ± 21 |
| 1 498 | Anisyl alcohol | Floral | 1672 ± 157 | 2179 ± 254 | 1396 ± 105 | 570 ± 72 | 593 ± 100 | 502 ± 30 | 4491 ± 484 | 202 ± 31 |

a. Odour descriptions were retrieved from AroChemBase (v 6, Alpha MOS), as well as from literature (Arn et al., 1998; Burdock, 2016)

In Table 8, a summary of the tentatively identified compounds is given, with inclusion of their odour descriptors. The cumulative peak area can be seen in Figure 12a, to represent the overall strength of the combined aroma for the 20 selected sensors. The greater the peak area, the greater the concentration of that compound in the aroma. Additionally, the fraction of the aroma that each odour descriptor accounted for, is represented in Figure 12b, to give an overview of the general differences between samples.

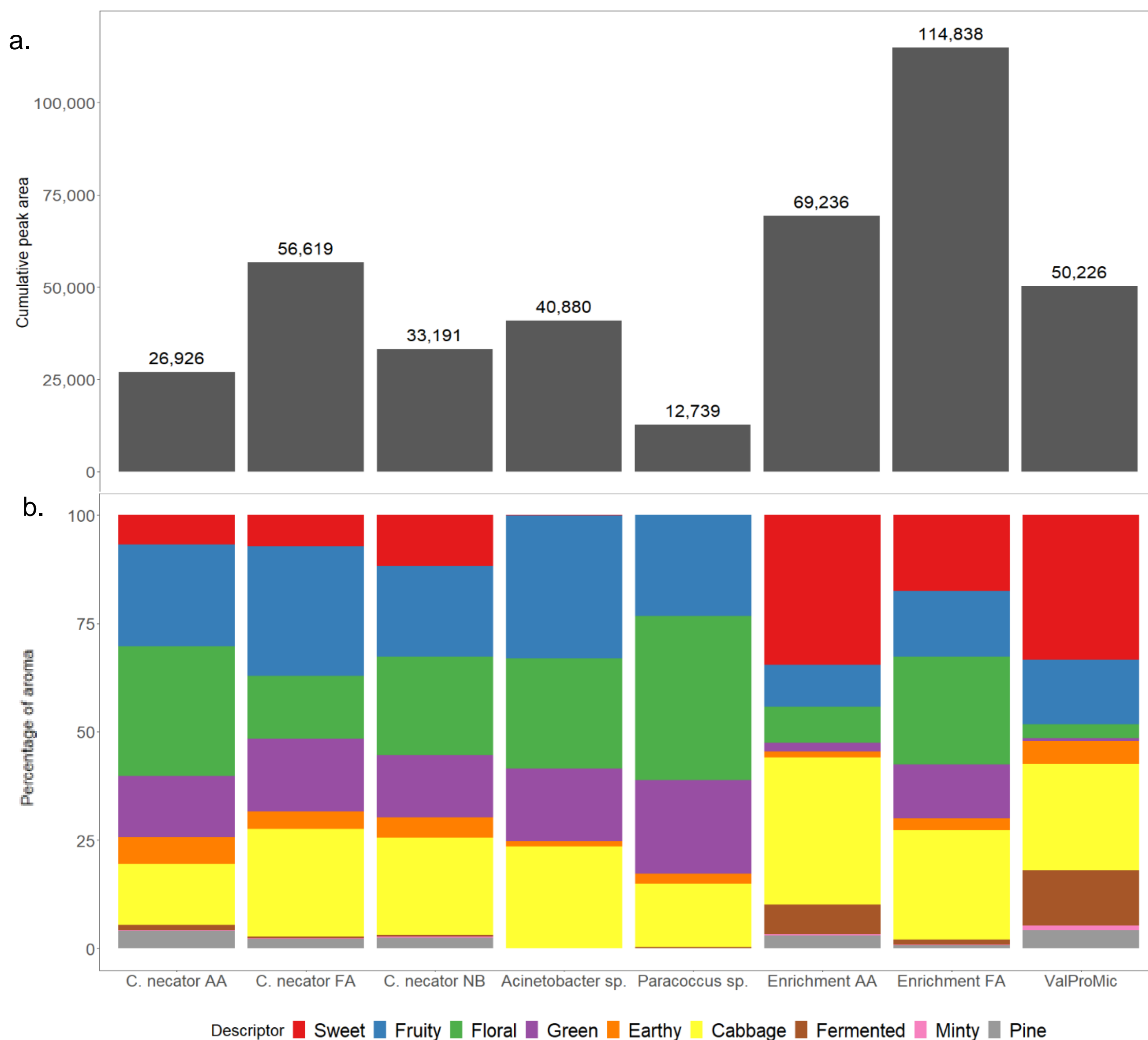



*Figure 12. a. Cumulative peak area of 20 selected peaks for C. necator, the isolates, i.e. Acinetobacter sp. and Paracoccus sp., and the enrichments grown on acetate and formate in batch growth. b. The fraction of the aroma that each odour descriptor accounted for. Peaks that exhibited the same odour descriptor were combined to a cumulative peak area. Abbreviations: AA= acetate, FA= formate, NB= nutrient broth.*

From Figure 12a, it is clear that the cumulative peak area of the 20 selected sensors was the highest for the enrichment on formate. This does not necessarily mean that the odour of this sample is more noticeable than that of the others, as not all compounds were

evaluated, but only those that accounted for the greatest variance. To this end, this high result does give an indication of a more distinct smell than the other samples. Interestingly, the strain that was isolated from this enriched culture, *Paracoccus sp.*, accounted for the lowest cumulative peak area, thus the least distinct odour. Moreover, both enrichments exhibited a higher peak area than the strains isolated from that culture. This could be because of the multitude of organisms present in a culture, that are each producing an array of VOC's, whereas a pure strain will only emit one set of VOC's.

For *C. necator*, it can be seen that the cumulative average area was more than double the amount when grown on formate than on acetate (Figure 12a), suggesting a stronger odour for the formate sample. The occurrence of the separate peaks was rather similar, resulting in a comparable odour composition, which had already been established by the relatively low Euclidian distance (Table 7). A few main differences were observed, that provided each sample its unique flavour. Benzyl alcohol, which gives a fruity aroma, was measured to be 4 times stronger in formate than in acetate. Dimethyl sulfoxide also exhibited a concentration 4 times higher than in acetate, giving more of a sulphurous, cabbage aroma to the formate-grown sample. These differences were clearly reflected in the aroma profile of the two samples (Figure 12b), with the fraction of fruity and cabbage aromas reasonably higher in the formate sample. The acetate sample exhibited more of a floral note, with 30% of the aroma being flower related odours. This was especially remarkable in comparison with the floral ratio for formate, which is about half of the acetate sample, since the absolute peak areas did not differ that much. However, since the cumulative peak area was much smaller for *C. necator* on acetate, these higher values accounted for a considerable amount of the aroma. In comparison with the odour of *C. necator* grown on nutrient broth, which was included as a reference, the profiles seem rather similar, which had already been established by the Euclidian distances (Table 7). These distances indicated a higher similarity between acetate and nutrient broth, although the aroma profile in Figure 12b seemed to contain elements from both the acetate and the formate grown samples, such as the higher ratio of cabbage compounds alike the formate sample and the lesser fruity compounds like the acetate sample. However, the cumulative peak area of the nutrient broth sample was comparable to the acetate sample, whereas formate exhibited a much larger peak area, which was likely the main cause of the higher Euclidian distance between the formate and nutrient broth samples.

The two isolates, *Acinetobacter sp.* and *Paracoccus sp.*, showed a considerable variation in cumulative peak area, with values for *Acinetobacter sp.* 4 times higher than for *Paracoccus sp.*, which also accounted for the smallest area out of all samples (Figure 12a). Assessing each peak separately, larger differences in peak area for benzyl alcohol,

6-decenal, dimethyl sulfoxide, and ethyl (methylthio)acetate can be noted (Table 8), rendering an aroma with more fruity and cabbage-like components to *Acinetobacter sp.* compared to *Paracoccus sp.*, as shown in Figure 12b. *Paracoccus sp.* was mainly characterised by floral components, due to the presence of anisyl alcohol. Yet, their overall aroma profiles were quite similar, especially for the first 8 components as these were present in very low quantities in both samples.

Lastly, both of the enrichments accounted for strong aroma profiles with high peak areas (Figure 12a), while these also exhibited the most differing profiles. The most notable is the difference for anisyl alcohol (floral), as the obtained peak area in the formate enrichment is almost 9 times higher than in the acetate culture. This large value explains the substantial floral fraction of the aroma of the formate enrichment. Further considerable differences were found for benzyl alcohol, 6-decenal, anisyl alcohol, geosmin, rheosmin, octyl caprylate, and dimethyl sulfoxide, representing a wide array of aroma descriptors (Table 8).

The comparison of the ValProMic aroma against all other samples shed a light on the greatly differing profile, as previously established with the Euclidian distances. Effectively, the aroma composition showed a considerable variation to the samples obtained from this study. The large ratios for fermented, minty, and sweet aromas were unprecedented. The presence of cyclohexanone in larger quantities, responsible for the minty notes, was unique to the ValProMic aroma. The very small amount of floral and green odour components also contributed to its more distinct aroma. From Figure 12, it seems that the acetate enrichment might be a close match, although the Euclidian distances prove otherwise. Based on this parameter, the similarity with *C. necator* on nutrient broth was higher, which does not seem likely based on the odour components in Figure 12. However, as only 20 components were evaluated, and these were the most varying compounds, it is quite possible that the overall aroma profile, as assessed in the PCA analysis and Euclidian distances, is in fact more similar.

Apart from these comparisons, it was also interesting to assess which odour descriptor had the largest impact on the aroma of each sample. The most dominant factor, as well as the percentage of the cumulative peak area that was represented by this descriptor, is presented in Table 9. In case the two most dominant descriptors exhibited very similar values, both were included.

*Table 9. Most dominant odour descriptors per sample. Abbreviations: AA= acetate, FA= formate, NB= nutrient broth.*

| **Sample** | **Odour descriptor** | |
|---|---|---|
| *C. necator* AA | Floral | 30.0 % |
| *C. necator* FA | Fruity | 29.9 % |
| *C. necator* NB | Floral | 22.7 % |
| | Cabbage | 22.4 % |
| *Acinetobacter sp.* | Fruity | 33.1 % |
| *Paracoccus sp.* | Floral | 37.9 % |
| Enrichment AA | Sweet | 34.6 % |
| | Cabbage | 34.0 % |
| Enrichment FA | Cabbage | 25.3 % |
| | Floral | 24.8 % |
| ValProMic | Sweet | 33.4 % |

The samples seemed to exhibit more fruity, floral, or sweet notes than any other odour group. It should be noted though that these odour descriptors were represented the most among the 20 assessed VOC's, with 6 entries for fruity flavours, 3 floral and 2 sweet. Naturally, the total peak area of several components accounting for the same descriptor is likely to be higher and thus more dominant in the end result. However, this does not mean that the samples exhibited a fruity or floral odour. The 20 assessed peaks only explain a minimal part of the aroma, as aroma is affected by many factors. Additionally, the peaks were not selected because they were the highest, and thus most dominant, but because they exhibited the most variance, which allowed to assess the most distinctive components

Lastly, it should be stressed once again that the obtained components were only identified tentatively, and it can thus not be guaranteed that the compounds that were identified here are the actual volatiles present in the sample.

# 7. Remark

Both the protein content and the protein productivity have been assessed simultaneously in this study, although they represent two different points of view. Naturally, for industrial microbial protein production, the ultimate goal is to achieve a sufficient protein yield. Now, this can be achieved in two ways: on the one hand by aiming at a high protein content and on the other hand by fast production, even though protein contents might be lower. Both systems will have pros and cons, but ultimately this choice will depend on the feasibility of high protein contents or high productivities.

# V. CONCLUSIONS AND FUTURE RESEARCH

The main objective of this research was to assess the influence of cultivation conditions on microbial protein and its flavour.

In the first phase, the goal was to examine the influence of the choice of micro-organism and carbon source on the protein content in the cell, as well as total protein concentration. This was evaluated by separate batch growth experiments, during which biomass concentration and protein concentration were monitored. The impact of each of these parameters could then be assessed through statistical analyses. Overall, both the micro-organism and the carbon source selection had a significant influence on the protein content and concentration. Moreover, the choice of the micro-organism had a more influencing impact on the protein levels than the change in carbon source. It could however not be predicted which selection of carbon source or micro-organism would result in the highest protein content, since only *C. necator* had been truly grown on both acetate and formate. During the experiments with the isolates and enrichments, the organisms grown on acetate differed from the ones grown on formate. As both the carbon source and the organism varied in these cases, they did not allow for a direct comparison, as opposed to the experiments with *C. necator*. In further research, the growth of strains supplied with several carbon sources could be included to be able to fully assess the influence of the carbon source on the protein. The use of different strains can also be of interest to assess which organism would be most suitable.

Next, the protein content was evaluated throughout the different phases of the bacterial batch growth. It was hypothesised that the protein content would reach a maximum during the exponential phase, because the protein production is linked to the growth rate through the abundance of ribosomes. However, this trend was not observed, as the protein content either increased continuously or exhibited a rather constant protein content. The protein content presented a sigmoid-shaped increase, with the fastest gain during the exponential phase, which lead to the conclusion that it was the protein production rate, rather than the protein content, that reached a peak during the exponential growth phase. This was also confirmed by the analysis of the protein productivity through time. The differing trend of the protein content through time was explained by the varying level of impact growth rate has on specific proteins, which cannot be predicted when assessing the whole proteome. Because of the link between protein production rate and growth rate, it was concluded that the growth rate was the most influential factor on protein content, rather than the growth phase.

The third part of this thesis involved the use of an electronic nose to assess the flavour of each sample. To our knowledge, this was the first time such a technique was applied for microbial protein and it was demonstrated that the eNose could be effectively used as a means to discriminate between the aromas of the 6 dried samples obtained in this study. The impact of the difference in micro-organism or carbon source on the aroma profile was also evaluated, concluding that the organism was the most influential of the two. Yet, this trend was not consistent through all samples, so it is possible that the metabolism applied by the organism might actually influence the volatile profile too, rather than the organism in se.

Lastly, an attempt was made to identify the volatile compounds present in the microbial protein aroma through the selection of 20 components that accounted for the highest variance between samples. As this identification was performed on the basis of Kovats retention indices calculated with experimental retention times, these components could only be identified tentatively. It is thus not guaranteed that these are actually present in the samples' aroma profiles. These components could also be linked to their respective aroma descriptors. From these tentative results, the formate enrichment exhibited the greatest cumulative peak area, and thus largest concentration of the 20 selected components, while *Paracoccus sp.* accounted for the smallest peak area.

The use of the eNose was a promising first attempt at unravelling the aroma of microbial protein and how it might be influenced by cultivation conditions. However, as the volatile components could not be identified surely, the use of more analytical techniques, such as mass spectrometry, would be a great asset in further research to identify volatile compounds and how these are affected.

For example, as micro-organisms have to allocate their resources to the production of several metabolites, one of them being protein versus growth, the protein content might affect the volatiles produced and thus the aroma profile of the samples (Goelzer et al., 2011). As the components were not surely identified in this study, the influence of this factor on the aroma profile was not researched here. Yet, assessing how aroma can be impacted would definitely be of great benefit to future industrial products, given that animals and consumers are very sensitive to the smell of products.

An additional field of interest could be the protein quality, i.e. amino acid composition, on the one hand how this is impacted by cultivation conditions, but on the other hand how the composition might account for differences in flavour.

# VI. REFERENCES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# VII. APPENDICES

## 1. Amino acid composition for various protein sources in literature

*Table 10. Average amino acid composition for protein from HOB (C. necator), yeast (S. cerevisiae), fishmeal, soybean meal, casein and the FAO standard scoring pattern for essential amino acids for children (>3y) and adults. Expressed as g/100 g protein.*

| Amino acid | HOB | Yeast | White fishmeal | Soybean meal | Casein | FAO standard |
|---|---|---|---|---|---|---|
| Alanine | 10.70 | | 6.31 | | 2.83 | |
| Arginine | 8.15 | 3.75 | 5.69 | 7.55 | 3.43 | |
| Aspartic acid | 9.34 | | 8.54 | | 6.76 | |
| Cystine | 0.30 | 1.10 | 0.97 | 1.32 | 0.34 | |
| Glutamic acid | 10.24 | | 12.79 | 18.40 | 21.20 | |
| Glycine | 7.84 | | 9.92 | | 2.14 | |
| Histidine | 1.94 | 2.40 | 2.16 | 2.48 | 2.50 | 1.60 |
| Isoleucine | 4.54 | 4.85 | 4.15 | 4.99 | 5.10 | 3.00 |
| Leucine | 8.61 | 7.20 | 6.89 | 7.69 | 8.80 | 6.10 |
| Lysine | 8.91 | 7.55 | 6.95 | 6.43 | 7.24 | 4.80 |
| Methionine | 1.55 | 1.75 | 2.60 | 1.31 | 2.56 | |
| Phenylalanine | 3.78 | 4.20 | 3.65 | 4.95 | 4.57 | |
| Proline | 2.13 | | 5.34 | | 10.31 | |
| Serine | 4.14 | | 4.75 | | 5.71 | |
| Threonine | 4.86 | 4.85 | 4.03 | 3.88 | 4.14 | 2.50 |
| Tryptophan | 1.16 | 1.20 | 1.07 | 1.26 | 1.14 | 0.66 |
| Tyrosine | 2.83 | 3.60 | 2.75 | 3.03 | 5.23 | |
| Valine | 7.32 | 5.60 | 4.84 | 5.08 | 6.34 | 4.00 |
| | | | | | | |
| SAA (Cys+Met) | 1.85 | 2.85 | 3.57 | 2.64 | 2.90 | 2.30 |
| AAA (Phe+Tyr) | 6.61 | 7.80 | 6.40 | 7.99 | 9.80 | 4.10 |
| References | [1] | [2] | [3] | [4] | [5] | [6] |

[1] (Calloway et al., 1969; Volova et al., 2010)

[2] (Garg et al., 1980; Najafpour, 2015)

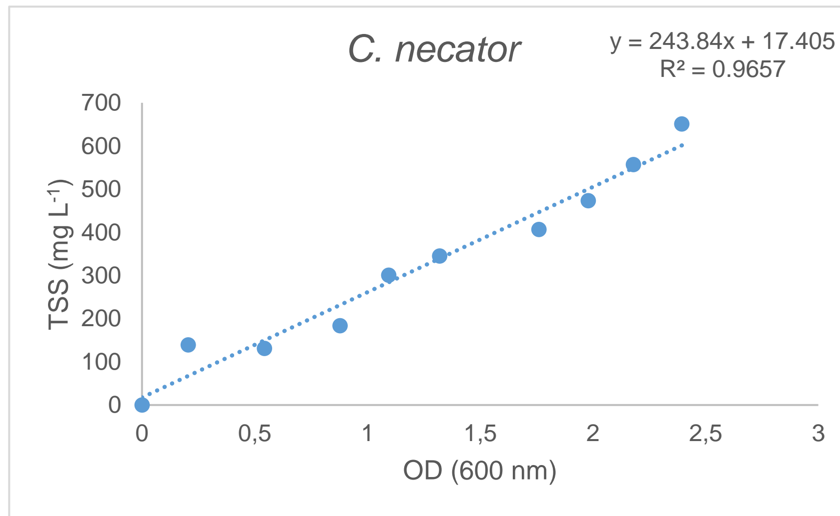
[3] (FAO, 1987; Garg et al., 1980; International Association of Fish Meal Manufacturers, 1970)

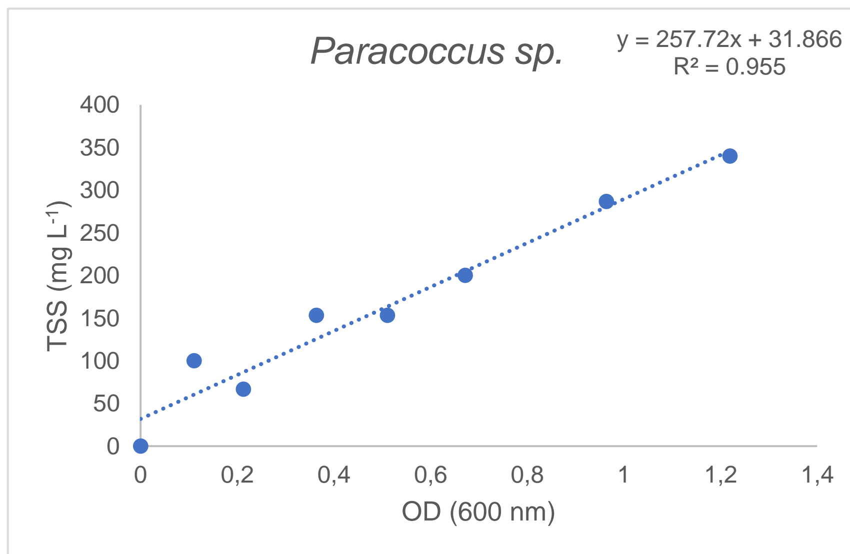
[4] (Banaszkiewicz, 2011; FAO, 1987; Garg et al., 1980; Kuiken et al., 1949)

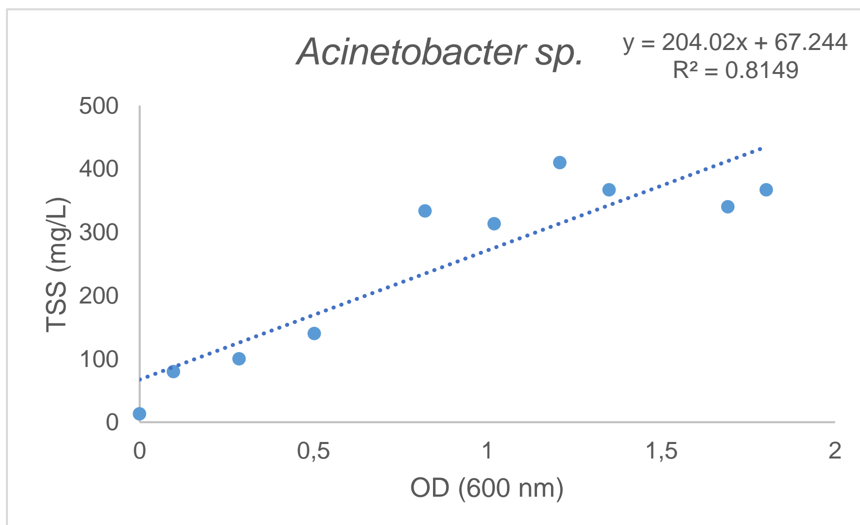
[5] (FAO, 1987; Rasmussen et al., 2008; Volova et al., 2010)

[6] (FAO, 2013a)

## 2. OD - TSS calibration curves for the pure cultures

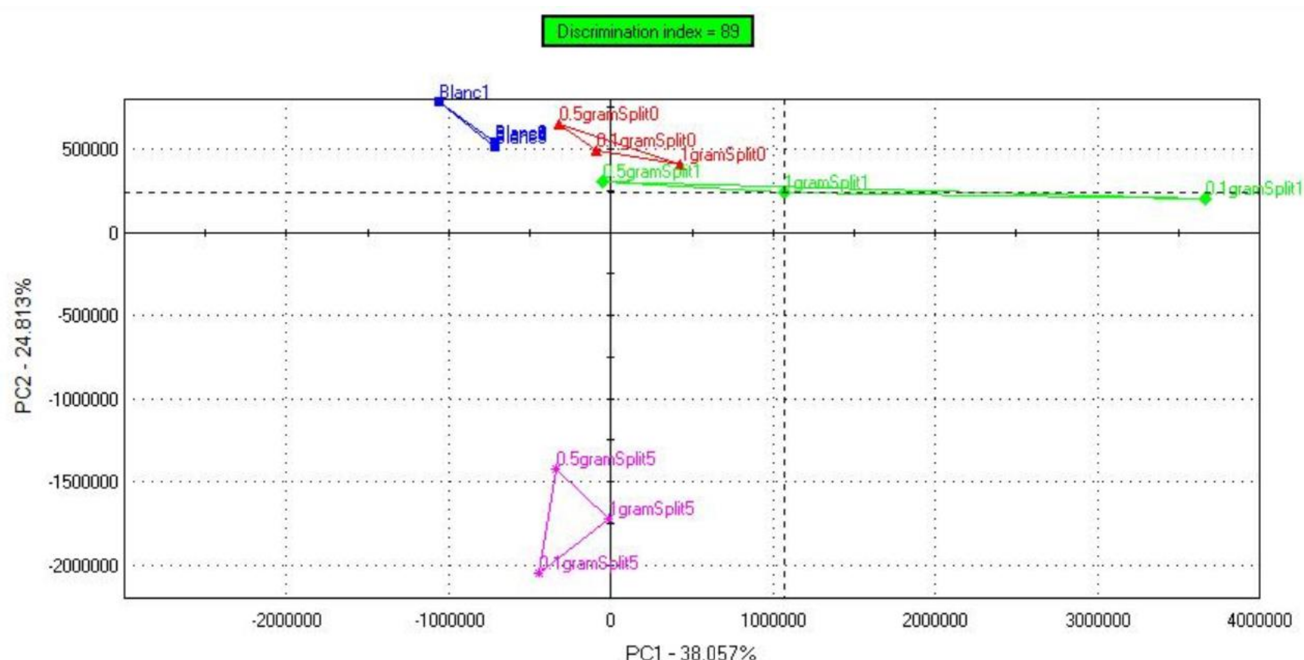


## 3. Results of the eNose method optimisation

For the optimisation of the eNose analysis parameters, three different split ratios of 0, 1, and 5 were tested, as explained in section III.6.2. Additionally, three different sample amounts were included, i.e. 0.1 g, 0.5 g, 1 g. Due to sample limitations, 0.1 g was the only feasible option, but the other sample size were included to ensure no information was lost due to the small sample. The aroma of the ValProMic biomass was then assessed using all 9 combinations, of which the results are presented in the PCA graph.




First, a positive discrimination index was observed, indicating sufficient discrimination power between each aroma profile. However, the blanc samples (blue), which were empty vials, seem rather close to the samples obtained with the Split 0 method (red) and the Split 1 method (green), whereas the samples that assessed with a split ratio of 5 mL min$^{-1}$ were further apart from the blancs. Additionally, the Split 1 samples showed a great variance between the three sample sizes, which could indicate a loss of peaks and thus information due to small sample. For these reasons, a split ratio of 5 mL min$^{-1}$ was chosen to be applied for all other eNose analyses conducted in this study.

## 4. Dried biomass for the eNose analyses

C. necator Formate | C. necator Acetate | C. necator Nutrient broth

Isolate Formate | Isolate Acetate

ValProMic Potato waste stream | Enrichment Acetate | Enrichment Formate

## *5.* DNA consensus sequences of *Acinetobacter sp.* and *Paracoccus sp.*

The consensus sequences for *Acinetobacter sp*. and *Paracoccus sp.* were obtained by PCR amplification with the primer pair 27F and 1492R, and subsequent Sanger sequencing.

*Acinetobacter sp.*

CTTACCATGCAAGTCGAGCGGGGAAGGGTAGCTTGCTACCTAACCTAGCGGCGGACGGGT

GAGTAATGCTTAGGAATCTGCCTATTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATA

CCGCATACGCCCTACGGGGGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGATGAGCC

TAAGTCAGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGT

CTGAGAGGATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG

CAGTGGGGAATATTGGACAATGGGGGGAACCCTGATCCAGCCATGCCGCGTGTGTGAAGA

AGGCCTTTTGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTCTTCTAGTTAATACCTAGG

ATGAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAA

TACAGAGGGTGCGAGCGTTAATCGGATTTACTGGGCGTAAAGCGTACGTAGGCGGCTTTT

TAAGTCGGATGTGAAATCCCTGAGCTTAACTTAGGAATTGCATTCGATACTGGGAAGCTA

GAGTATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAG

GAATACCGATGGCGAAGGCAGCCATCTGGCCTAATACTGACGCTGAGGTACGAAAGCATG

GGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTCTACTAGCCGT

TGGGGCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTAC

GGTCGCAAGACTAAAACTCARATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTG

GTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATAGTAAGAACTTTCCAG

AGATGGATTGGTGCCTTCGGGAACTTACATACAGGTGCTGCATGGCTGTCGTCAGCTCGT

GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTTTCCTTATTTGCCAGCG

GGTTAAGCCGGGAACTTTAAGGATACTGCCAGTGACAAACTGGAGGAAGGCGGGGACGAC

GTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGTCGGTACAAA

GGGTTGCTACCTAGCGATAGGATGCTAATCTCAAAAAGCCGATCGTAGTCCGGATTGGAG

TCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGAATGCCGCGGT

GAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAATTTGTTGCACCAG

AAGTAGGTAGTCTAACCGCAAGGAGGACGC

*Paracoccus sp.*

TGCAAGTCGAGCGAGACCTTCGGGTCTAGCGGCGGACGGGTGAGTAACGCGTGGGACTGT
GCCCTTCTCTACGGAATAGCCTCGGGAAACTGGGAGTAATACCGTATACGCCCCTTGGGG
GAAAGATTTATCGGAGAAGGATCAGCCCGSGTTGGATTAGGTAGTTGGTGGGGTAATGGC
CTACCAAGCCGACGATCCATAGCTGGTTTGAGAGGATGATCAGCCACACTGGGACTGAGA
CACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATCTTAGACAATGGGGGCAACCCT
GATCTAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTCAGCTGGG
AAGATAATGACGGTACCAGCAGAAGAAGCCCCGGCTAACTCCGTGCCAGCAGCCGCGGTA
ATACGGAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGACCA
GAAAGTTGGAGGTGAAATCCCAGGGCTCAACCTTGGAACTGCCTTCAAAACTATTGGTCT
GGAGTTCGAGAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGA
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CAAGTCCTCATGGCCCTTACSGGTTGGGCTACACACGTGCTACAATGGTGGTGACAGTGG
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GGAATCGCTAGTAATCGCGGAACAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACA
CACCGCCCGTCACACCATGGGAGTTGGTTCTACCCGACGGCCGTG