Microbial production of chemical building blocks from lignin

a Master Thesis Project

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Abstract

Plastics, derived from fossil fuels, have raised considerable environmental concerns. This has prompted the search for sustainable and safer plastic precursors alternatives. One alternative explored in the present study is vanillin, a phenolic compound derived from lignin, an abundant and renewable natural polymer. Vanillin and its derivatives have shown promise as eco-friendly substitutes for conventional industrial chemicals. The present study focused on the microbial production of chemical building blocks from vanillyl amine and vanillic acid using recombinant yeast, with a specific focus on the evaluation of amide-forming enzymes. The study also involved the screening of various co-substrates to optimize the production of vanillyl amine and vanillic acid. Our findings revealed that the choice of co-substrate significantly influenced the production of vanilloids, with specific co-substrates favoring the production of specific vanilloids.

Foreword

It is with great pleasure and a sense of accomplishment that I present my master thesis, the culmination of an exploration into sustainable alternatives for traditional industrial chemicals. Plastics, derived from fossil fuels, have long been a source of environmental concern, propelling the search for eco-friendly substitutes. This study delves into the potential of vanillin, a phenolic compound derived from lignin, a renewable natural polymer, as a promising candidate for this purpose. This master thesis was performed from the 15th of May until the 6th of November, and lasted for 25 weeks. All practical experiments were performed at Kemicentrum, Sölvegatan 39, 223 62 Lund, and equipment was provided by the Division of Applied Microbiology.

This academic journey would not have been possible without the support, guidance, and collaboration of numerous individuals. First, I extend my sincere gratitude to Magnus Carlquist, whose unwavering support and invaluable guidance have been instrumental in steering this research. Their expertise and encouragement have been a constant source of inspiration.

I would also like to express my appreciation to Isabela Mendes Bonfim, Matilda Johansson, and Marie Gorwa-Grauslund whose insights, discussions, and contributions have enriched the depth and scope of this work. The collaborative environment fostered at Applied Microbiology has played a pivotal role in shaping the methodology and approach of this study.

The challenges encountered along this research journey have been valuable learning experiences. Despite setbacks in the biosynthesis of chemical building blocks from vanillyl amine and vanillic

acid, the study has opened avenues for future exploration, positioning yeast as a promising platform for amide-forming processes.

1.Introduction

1.1 Exploring the potential biosynthesis of bisguaicol amide

Plastics are widely used in our society, but their production has a significant environmental impact. Most of today's plastics come from non-renewable fossil fuels, have a long lifespan, and their production and use result in a significant amount of greenhouse gases (Cabernard et al, 2021). Therefore, there is a need for greener plastics made from sustainable and renewable sources, that are biodegradable and have a shorter lifespan. Such greener plastics can significantly reduce the environmental impact while meeting the societal needs (Moshood et al, 2022).

One chemical that poses an environmental challenge is bisphenol A (BPA). BPA is a vital precursor in producing synthetic polymers, including epoxy resins, polycarbonates, and benzoxazine compounds (Konieczna et al, 2015). Currently used in the production of polycarbonate plastics and consumer goods like food and drink containers, BPA is derived from fossil fuels and has been linked to negative health effects due to its endocrine system disruption (Hafezi and Abdel-Rahman, 2019). This environmental and health impact has resulted in a growing need for more sustainable and safe alternatives to replace BPA in response to the increasing demand for eco-friendly chemicals.

Vanillin is a widely available phenolic compound that is commonly derived from lignin through a chemical oxidation process (Banerjee and Chattopadhyay, 2020). Lignin is an abundant natural polymer, cost-effective, and renewable, making it an attractive alternative to products derived from fossil fuels (Becker and Wittman, 2019). Lignin derivatives, including vanillic acid, vanillyl alcohol, and vanillyl amine, can be obtained from vanillin and used as starting materials for producing bisphenols. By using vanillin as a raw material, we can take advantage of its ample availability, renewability, and affordability to develop sustainable and environmentally friendly substitutes for conventional industrial chemicals (Banerjee and Chattopadhyay, 2020).

To assemble two phenol groups into a single molecule, various approaches utilizing vanillin derivatives have been documented. Among these, a study accomplished the chemical synthesis of bisguaiacol amide (BGA) from vanillyl amine and vanillic acid (Sun et al, 2023). BGA, in contrast to BPA, is a renewable and non-toxic bisphenol while sharing the same properties as BPA, which underscores the significance of replacing it with a biobased polymer like BGA. (Sun et al, 2023). Nevertheless, the microbiological production of BGA remains an unexplored field. Microbiological production is of particular interest due to its potential to use renewable resources and the ability through metabolic engineering to further improve the yield of a target compound. Another advantage lies in its avoidance of heavy metals, organic solvents, and strong acids and

bases, enabling an environmentally friendly synthetic process (Du et al, 2011). Although no studies have been conducted on the microbial conversion of vanillic acid and vanillyl amine to BGA, a study on *S. cerevisiae* to produce capsaicinoid nonivamide has developed a promising platform that could potentially be adapted for BGA synthesis (Muratovska et al, 2022). This progress was made possible by utilizing a combination of amide forming NAT (*N*-acetyltransferase) and CL (CoA ligase) enzymes. Additionally, previous studies have shown that amination reactions can produce strong and durable polymers, highlighting the potential for BGA to be used in high-performance materials (Chawla and Chawla, 2018). Therefore, the potential synthesis of bisguaiacol amide (BGA) from vanillic acid and vanillyl amine was investigated in this project (Figure 1). This approach could provide a sustainable and cost-effective route for BGA production.

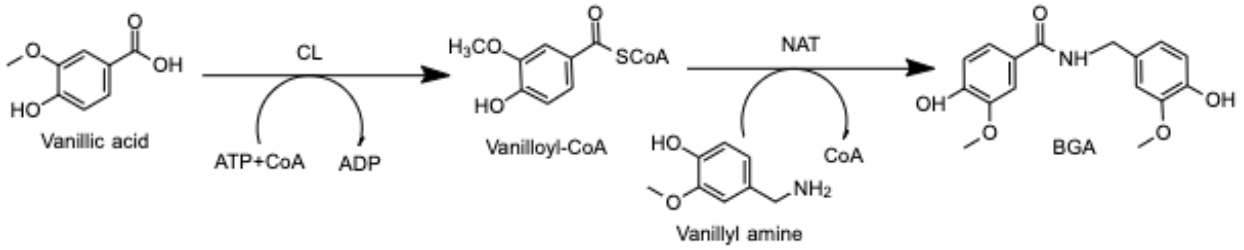

Figure 1. Potential bioconversion route of vanillic acid and vanillyl amine into bisguaiacol amide (BGA) using the *S. cerevisiae* strains TMBNM020-TMBNM031 developed by Muratovska et al (2022). CL, CoA-ligase. NAT, *N*-acyltransferase.

1.2 Impact of co-substrates in the bioconversion of vanillin

When considering the applicability of lignin derived compounds, the bioconversion of vanillin, a key precursor for different vanilloids, is of significant interest. Vanillic acid and vanillyl amine are essential intermediates in the prospective BGA synthesis pathway. The conversion of vanillin into vanillyl amine and vanillic acid is subject to various factors, including the choice of co-substrate. Co-substrates are important because they have a direct connection to the redox and energy metabolism, affecting, among others, the production of NADH, NAD^+, and NADPH, which, in turn, influences the production of vanillyl amine, vanillic acid, and the by-product vanillic alcohol (Figure 2). In the present study a screening of different co-substrates using a *S. cerevisiae* mediated bioconversion was carried out. This screening aimed to elucidate the influence of diverse co-substrates on the selective conversion of vanillin into vanillyl amine and vanillic acid while mitigating the formation of undesirable side products such as vanillyl alcohol.

The yeast strain TMBNM033, employed in this investigation, has been genetically engineered to express multiple copies of an amine transaminase from *Chromobacterium violaceum* (Cv-TA). Additionally, it incorporates an NADH-dependent alanine dehydrogenase from *Bacillus subtilis* (BsAlaDH) and has its alcohol dehydrogenase 6 gene deleted (Figure 2). These genetic

modifications have been strategically designed to enable efficient reductive amination of vanillin, providing a robust enzymatic platform for the bioconversion process (Muratovska and Carlquist, 2023).

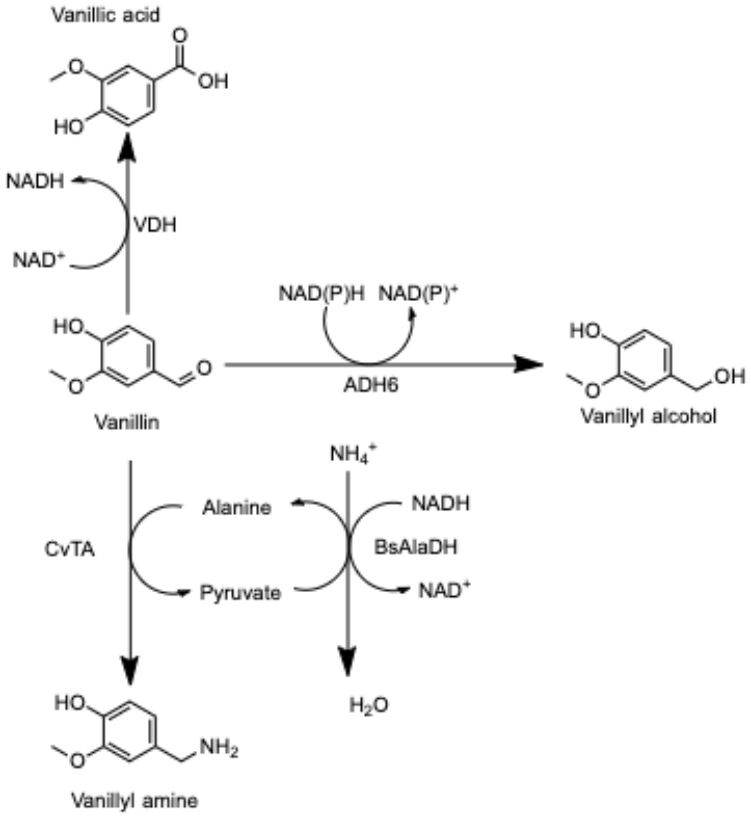

Figure 2. Production of vanillyl amine, vanillyl alcohol, and vanillic acid from vanillin by the engineered yeast strain TMBNM033 overexpressing the amine transaminase from *Chromobacterium violaceum* (CvTA) and the alanine dehydrogenase from *Bacillus subtilis* (BsAlaDH), and with deleted alcohol dehydrogenase gene (*ADH6*).

1.3 Aim

The aim of this project was:
i) To evaluate NAT-CL cascades for bisguaiacol amide (BGA) production from vanillyl amine and vanillic acid.

ii) To evaluate the effectiveness of various co-substrates in the bioconversion of vanillin to produce vanillyl amine and vanillic acid.

2. Materials and Methods

2.1 Strains and media

S. cerevisiae strains were maintained on Yeast Extract-Peptone-Dextrose (YPD) medium (20 g/L peptone, 10 g/L yeast extract and 20 g/L glucose). Defined mineral medium buffered at pH 6.5 (Verduyn et al.,1992) was used for the bioconversion assays. For BGA production, the 12 strains TMBNM20-31 (previously described in Muratovska et al 2022) were evaluated. These strains have integrated a combination of NAT (PaAT, CaAT, SiAT, and AT3) and CL (PhCL, ippF, and ACS) enzymes. For the co-substrate screening assays, the strain TMBNM033 was used for vanillin bioconversion.

2.2 Toxicity assay

A toxicity assay was performed by cultivating yeast cells in the presence of different concentrations (0, 0.1, 2.5, 5, and 10 mM) of vanillyl amine and vanillic acid separately in YPD media. The pre-cultures were prepared from a single colony (strain CEN.PK 113-7D) and grown in 5 mL of YPD media overnight. Next, ~70 mL serum vials containing 20 mL of media were inoculated for an initial optical density (OD) of 0.2 and kept in a water bath at 30 °C with constant agitation using magnetic stirrers. A periodic sampling with syringes at hourly intervals was performed for 48 hours.

2.3 Bioconversion of aromatics to BGA

Thirteen pre-cultures were prepared from a single colony (strains TMB MM20-31 and control strain CEN.PK 113-7D) in 5 ml of YPD media and grown overnight. Shake flasks with 25 mL defined mineral medium (Verduynet al., 1992) containing glucose (20g/L) were inoculated to initial optical density (OD620nm) of ~0.2 and incubated at 30°C and 180 rpm for 24–26 h to generate biomass. Subsequently, yeast cells were harvested by centrifugation at 4000 g for 10 min (Eppendorf Centrifuge 5810 R, Germany) washed and re-suspended for a final OD620nm of ~2 in buffered defined mineral medium (pH 6.5) supplemented with vanillyl amine (5 mM) and vanillic acid (5 mM) and glucose (10g/L) as a co-substrate. Whole-cell bioconversions were performed under oxygen-limited conditions in ~70 mL serum vials containing 25 mL of medium and incubated at 30°C in and 180 rpm for 120 hours. The vials were sealed with rubber lids which were then pierced by two 2.5mL sterile syringes, one for sampling and one filled with cotton working as an air vent.

2.4 Bioconversion of vanillin

Pre-cultures duplicates were prepared from a single colony each by inoculating 5 mL or 200 mL of YPD media for growing or resting cells assays, respectively. The cultures were then placed in a shaking incubator, set at 30°C and 180 rpm for 24 hours. Next, yeast cells were harvested by centrifugation at 4000 g for 10 min (Eppendorf Centrifuge 5810 R,Germany) and re-suspended in in defined mineral media (Verduyn et al., 1992) containing glucose (20 g/L) and incubated at 30°C and 180 rpm for 24 hours. The yeast cells were then centrifuged at 4000 g for 10 min, washed with sterile water and resuspended in 20 ml of buffered defined mineral medium (pH 6.5) supplemented with 5 mM vanillin and 10 g/L of the specified co-substrate (glucose, ethanol, glycerol, mannose, maltose, galactose, raffinose, ribose, sorbitol, sucrose, 2,3-butanediol, formic acid, glutamic acid, xylose, lactate, mannitol, rhamnose, and without co-substrate (control)). Growing and resting cells assays had a starting OD620nm of ~0.1 and ~5, respectively. Whole-cell bioconversions were performed under oxygen limited conditions in ~70 mL serum vials, incubated at 30°C and 180 rpm for 48 respectively 120 hours. The vials were sealed with rubber lids which were then pierced by two 2.5 mL sterile syringes, one for sampling and one filled with cotton working as an air vent.

2.5 Analytical methods

Samples were collected with a syringe regularly from cell cultures for analysis of yeast cell concentration and extracellular metabolite analysis. Yeast cell concentration was estimated by measuring optical density at 620 nm (OD620nm) with a spectrophotometer (Ultrospec 2100 pro UV/Visible spectrophotometer, Amersham Biosciences, Buckinghamshire, United Kingdom). For vanilloid analysis, the cell suspensions were centrifuged, and the supernatants were collected and subjected to High-Performance Liquid Chromatography (HPLC) analysis.

Reverse-phase chromatography was performed using a Select C18 column (4.6 × 150 mm) with mobile phase consisting of 0.1% trifluoroacetic acid (TFA; A) and acetonitrile (B) with a Waters HPLC system (Waters Binary HPLC pump 1525, UV/Vis detector 2489, Autosampler 2707, All Waters Corporation, Milford, USA). The analysis method employed was isocratic with 65% A and 35% B for 10 minutes, with a flow rate of 1 mL/min. Detection was performed at a wavelength of 281 nm at room temperature. For the quantification of compounds in the supernatants, standard curves were prepared with concentrations of 0, 0.05, 0.1, 0.25, 1, 5, and 8 mM of vanillin, vanillic acid, vanillyl amine, and vanillyl alcohol.

It is important to note that bisguaiacol amide (BGA) lacks a standard compound in which its presence can be directly measured. Therefore, the detection of BGA in the samples was approached through an indirect method, evaluating the consumption of the substrates (vanillyl amine and vanillic acid) and the appearance of new peaks in comparison to the control condition.

To achieve this, vanillyl amine and vanillic acid standards were employed as reference compounds.

2.6 Calculations

To determine the volume needed to reach a specific OD, equation 1 was used, where OD1 stands for the initial OD measurement, V1 is the volume required for transfer, OD2 represents the target OD value, and V2 is the final volume.

$$OD_1 \cdot V_2 = OD_2 \cdot V_2 \quad (1)$$

Yield is defined as moles of product (vanillyl amine/vanillic acid) divided by mol consumed substrate (vanillin). Relative ratio of vanillyl amine and vanillic acid is defined as mol product (vanillyl amine or vanillic) divided by moles by-product (vanillyl amine/vanillic acid + moles vanillyl alcohol.

3. Results

3.1 Evaluation of NAT and CL enzymes for BGA production

To evaluate the bioconversion of vanillin to BGA, a screening of a library with 12 different strains was conducted. These strains were previously engineered to have various combinations of amide-forming NAT (*N*-acyltransferase) and CL (CoA ligase) enzymes, and as well acyltransferase AT3 and the CoA-ligase AC, as seen in Table 1. The CL enzymes exhibit activity towards aliphatic carboxylic acids, specifically PhCL (4-coumarate: CoA ligase from *Petunia hybrida*) and IpfF (Ibuprofen CoA ligase from *Sphingomonas sp*. Ibu-2). The NAT enzymes are active in forming amides, particularly with aromatic amines such as 4-(aminomethyl) benzonitrile, as well as various thioesters, especially aliphatic CoA-esters. The NAT enzymes included PaAT (Arylamine *N*-acetyltransferase from *Pseudomonas aeruginosa*), CaAT (Tyramine *N*-(hydroxycinnamoyl) transferase from *C. annuum*), and SlAT (*N*-hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl) transferase THT1-3 from *Solanum lycopersicum*). The TMBMN020-031 strains have previously been employed for the production of nonivamide using vanillyl amine and nonanoic acid as substrates. As depicted in Figure 3, it is notable that the key distinction between nonivamide and BGA lies in the substitution of the amide acid component with a guaiacol moiety.

Table 1.Strains TMBNM020-31 used in the bioconversion of vanillin to BGA .Enzymes screened were PhCL (4-coumarate: CoA ligase from *Petunia hybrida*), IpfF (CoA ligase from *Spingomonas sp*. Ibu-2), CaAT (Tyramine *N*-hydroxycinnamoyl transferase from *Capsicum annuum*), SlAT (*N*-hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl) transferase THT1-3 from *Solanum lycopersicum*), PaAT (Arylamine *N*-acetyl-transferase from *Pseudomonas aeruginosa*), acyltransferase AT3 and the CoA-ligase ACS.

Strain	Gene combination
TMBNM031	PhCL + PaAT
TMBNM021	PhCL + CaAT
TMBNM022	PhCL + SIAT
TMBNM023	PhCL + AT3
TMBNM024	IpfF + PaAT
TMBNM025	IpfF + CaAT
TMBNM026	IpfF + SIaAT
TMBNM027	IpfF + AT3
TMBNM028	ACS + PaAT
TMBNM029	ACS + CaAT
TMBNM030	ACS + SIAT
TMBNM020	ACS + AT3

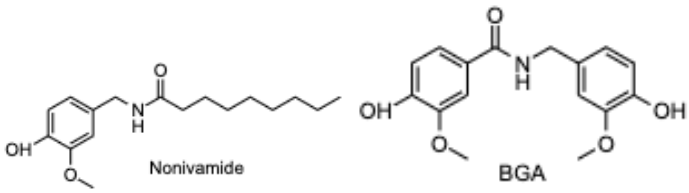

Figure 3. Picture of nonivamide and bisguaiacol amide

A schematic Figure of the experimental setup can be seen in Figure 4. Analysis was carried out using HPLC to detect BGA and spectrophotometry to measure cell concentration to assess whether the strains and their gene combinations exerted any impact on cell growth.

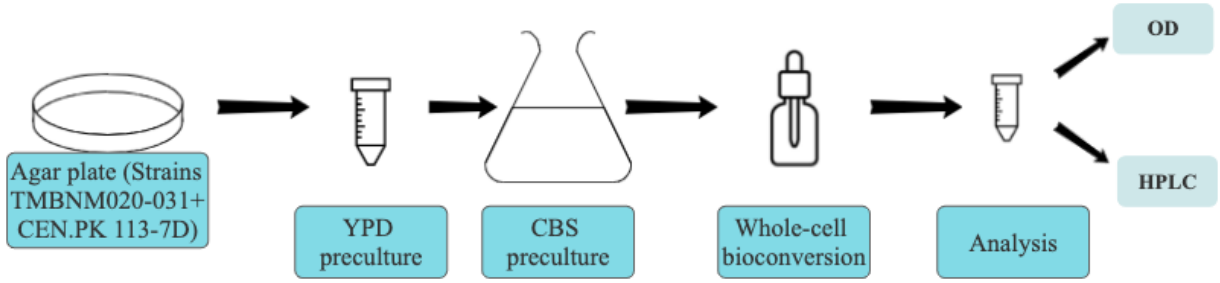

Figure 4. Overview of the experimental setup used in the study. TMBNM020-31 and the control strain CEN.PK 113-7D were obtained from YPD agar plates and incubated in 5 mL of YPD medium overnight. Subsequently, 25 mL of CBS media was inoculated and incubated overnight. Vials were then inoculated with an OD of 2.0 and supplemented with 5 mM of vanillyl amine and vanillic acid, and 10 g/L glucose. The experiment was carried out over a period of six days, during which High-Performance Liquid Chromatography (HPLC) and OD measurements were conducted daily on the supernatant to analyze the progress of the experiment.

3.1.1 Evaluation of inhibitory effects of vanillyl amine and vanillic acid on *S. cerevisiae*

To explore the suitability of *S. cerevisiae* as a potential platform host for BGA production and to determine suitable concentrations of vanillyl amine and vanillic acid, a series of growth inhibition experiments were conducted. The used strain was CEN.PK113-7D, which is a well-characterized and genetically stable MATα prototrophic strain of *S. cerevisiae*.

It was observed that vanillyl amine did not exhibit any inhibitory effects on *S. cerevisiae* up to the investigated concentration of 10 mM, whereas vanillic acid growth slightly declined above 5 mM (Figure 5). However, it is important to note that it was not possible to conduct a growth inhibition experiment to assess the effect of BGA on *S. cerevisiae* due to the unavailability of a BGA commercial standard. However, similar compounds such as bisphenol A (BPA) has been shown to significantly decrease the growth of *S. cerevisiae* at concentrations as low as 0.1 mM (Ďurovcová et al, 2021). Therefore, the concentration of 5 mM for vanillyl amine and vanillic acid was selected for subsequent experiments. This selection was based not only on the observation that the vanillic acid optical density began to decline at 5 mM but also due to solubility issues that arose after reaching 5 mM concentration.

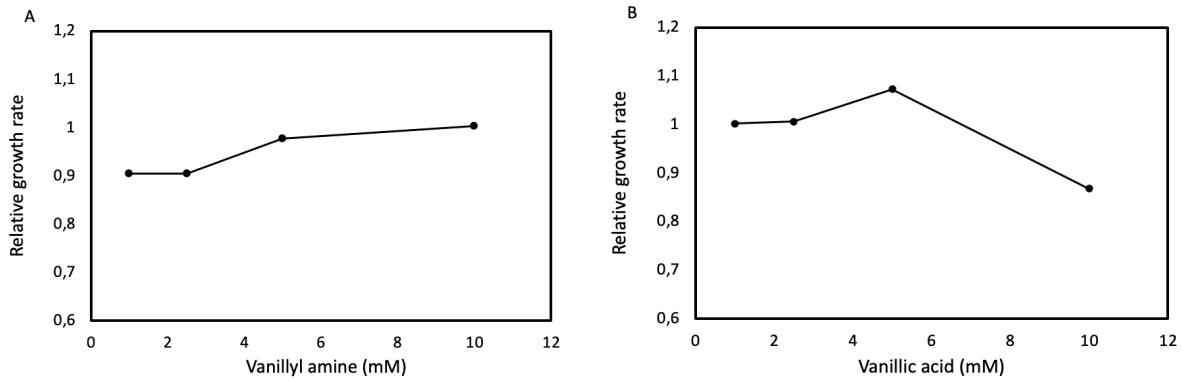

Figure 5. Relative growth of the control strain CEN.PK 113-7D in YPD media containing glucose (20 g/L) supplemented with 1-10 mM of vanillyl amine (A) or vanillic acid (B) after 48 hours. The cells were grown in serum vials in oxygen-limited conditions.

3.1.2 Library screening for growth

To assess the impact of the NAT and CL enzymes in the strains TMBNM020-31 on cell growth, a growth analysis was conducted and compared to the control strain CEN.PK 113-7D on glucose and in the presence of 5 mM vanillic acid and 5 mM vanillyl amine. The results revealed no discernible differences between all TMBNM020-31 strains and the control strain CEN-PK 113-7D, indicating that the gene combinations had no observable effect on the growth..

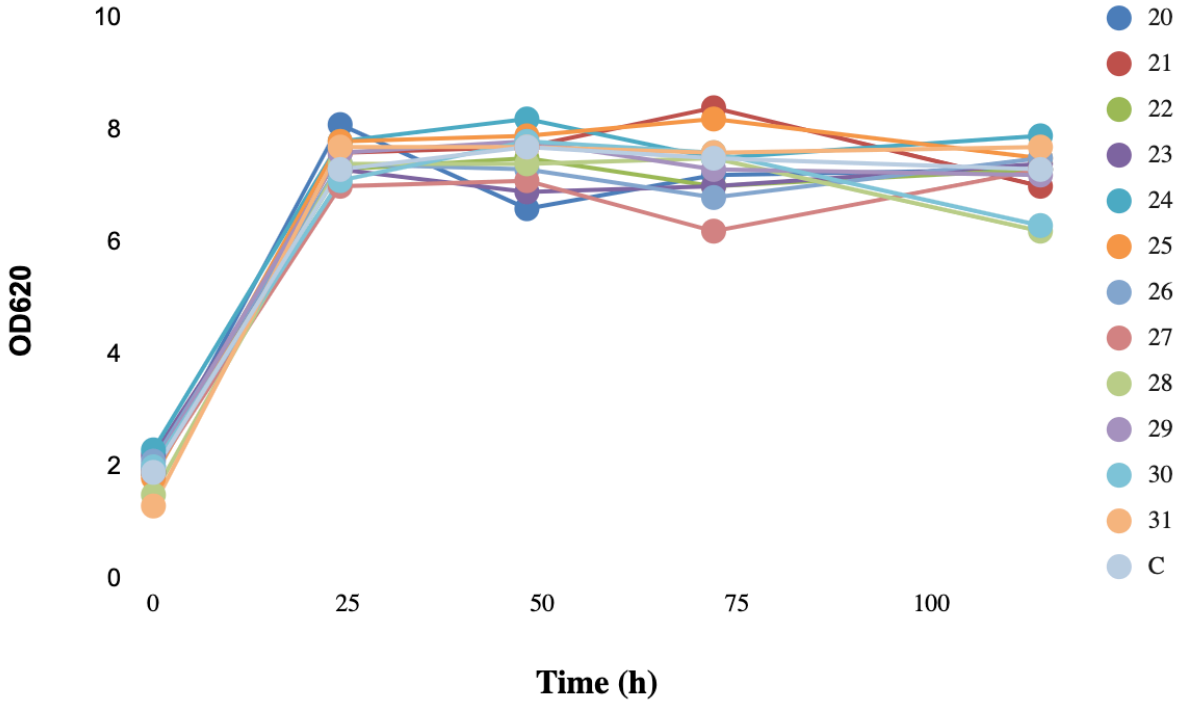

Figure 6. Growth of strains TMBNM020-031 (denoted as 20-31) and CEN.PK113-7D (C) in CBS media containing 5 mM vanillyl amine, 5 mM vanillic acid, and 10 g/L glucose as co-substrate.

3.1.3 Evaluation of NAT-CL combinations for vanillyl amine and vanillic acid conversion

The primary objective of the HPLC analysis was to detect if there had been any consumption of vanillyl amine and vanillic acid (Figure 7). A decrease in the concentration of vanillyl amine and vanillic acid in the samples could suggest their utilization for the synthesis of BGA. However, there were no significant differences when comparing all the strains vanillyl amine and vanillic acid consumption with the control. The small observed differences could instead be due to biological variation as only one replicate was made. In some strains there was an increase in

vanillyl amine and vanillic acid, which means that the method used was not perfect and that there was no bioconversion and very little metabolic activity.

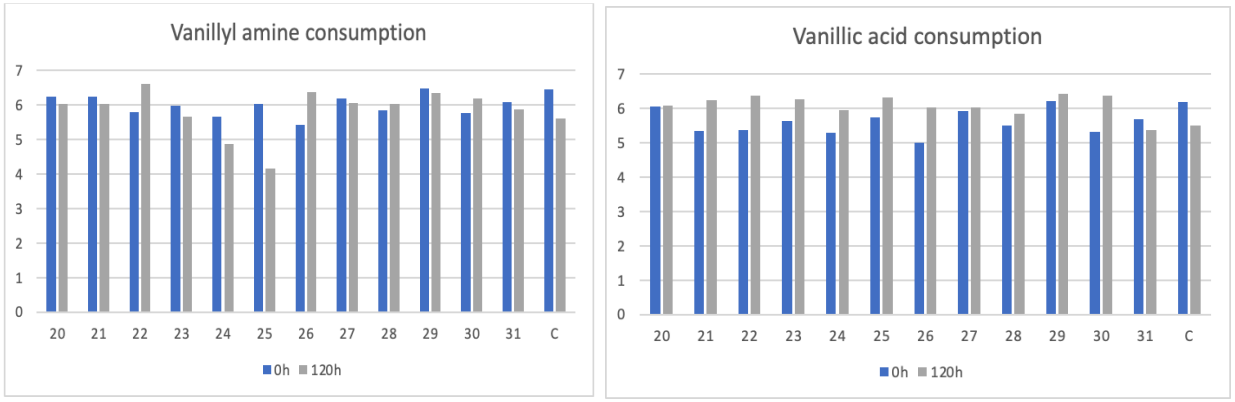

Figure 7. Vanillyl amine and vanillic acid consumption for TMBNM020-31 and control strain CEN-PK 113-7D. Blue bar represents the measured initial concentration of vanillyl amine and vanillic acid, and the gray bar the final concentration. Start OD 2 in CBS media containing 5 mM vanillyl amine, 5 mM vanillic acid, and 10 g/L glucose as co-substrate. Analyzed in HPLC.

The HPLC chromatograms were also analyzed to see if any new peaks emerged, as this could indicate the formation of BGA. However, the absence of a standardized reference for bisguaiacol amide (BGA) presented a significant challenge in the accurate identification of BGA in the experimental samples. Upon conducting the analysis, no discernible BGA was detected in either of the yeast strains as seen in Figure 8. Specifically, no new peaks emerged in the chromatograms that could correspond to the presence of BGA. A small peak was detected after 8.20 minutes; however, this peak was present in all strains (including the control) and the standards.

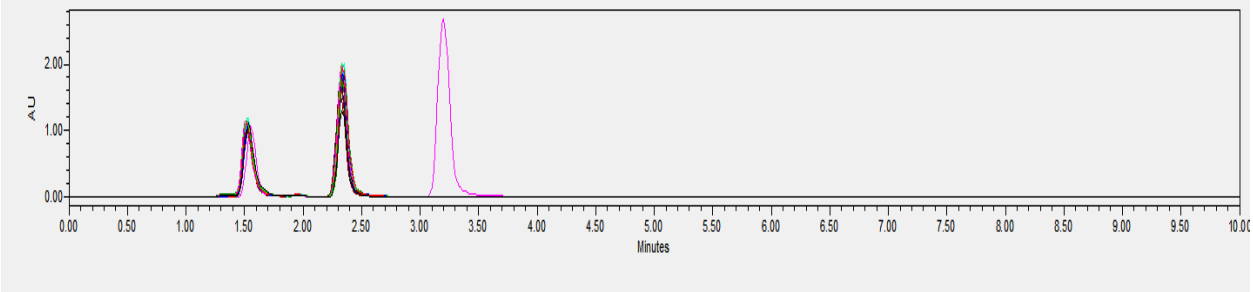

Figure 8. HPLC chromatogram for strains TMBNM20-TMBNM31 + control strain (CEN.PK 113-7D) at time 0h and after 6 days. First peak is vanillyl amine, second peak is vanillic acid, and the third peak is vanillin.

3.2 Evaluation of co-substrates impact on vanillyl amine and vanillic acid production

To investigate the influence of co-substrates in the bioconversion of vanillin into different vanilloids, 10 different co-substrates were evaluated on their impact on growth and the production of vanillin derivatives. The goal was to maximize the yield of vanillyl amine and vanillic acid while minimizing the formation of vanillyl alcohol in *S. cerevisiae* strain TMBNM033 overexpressing the amine transaminase gene from *Chromobacterium violaceum* (CvTA) and the alanine dehydrogenase gene from *Bacillus subtilis* (BsAlaDH), and with deleted alcohol dehydrogenase gene *ADH6* (cf. Figure 2).

The selection of co-substrates was based on their theoretical yield of NADH and NAD^+ when metabolized by *S. cerevisiae*. In addition these co-substrates should exhibit non-toxic properties towards yeast cells and be readily available in stock for experimental purposes. Accordingly, 10 co-substrates were selected (Table 2). An overview over the metabolic map when these co-substrates are metabolized can be seen in Figure 9.

Table 2. The theoretical yield of NADH and NAD+ alongside the degree of reduction for the ten co-substrates tested. These values are calculated based solely on the metabolic processing of each co-substrate prior to their entry into the tricarboxylic acid (TCA) cycle.

Co-Substrate	Theoretical yield of NADH	Theoretical yield of NAD^+	Degree of reduction
Glucose	2	0	24
Mannose	2	0	24
Galactose	2	0	24
Ribose	2	0	20
Maltose	4	0	48
Sucrose	4	0	48
Raffinose	6	0	72
Sorbitol	2	1	26
Glycerol	3	0	20
Ethanol	2	0	12

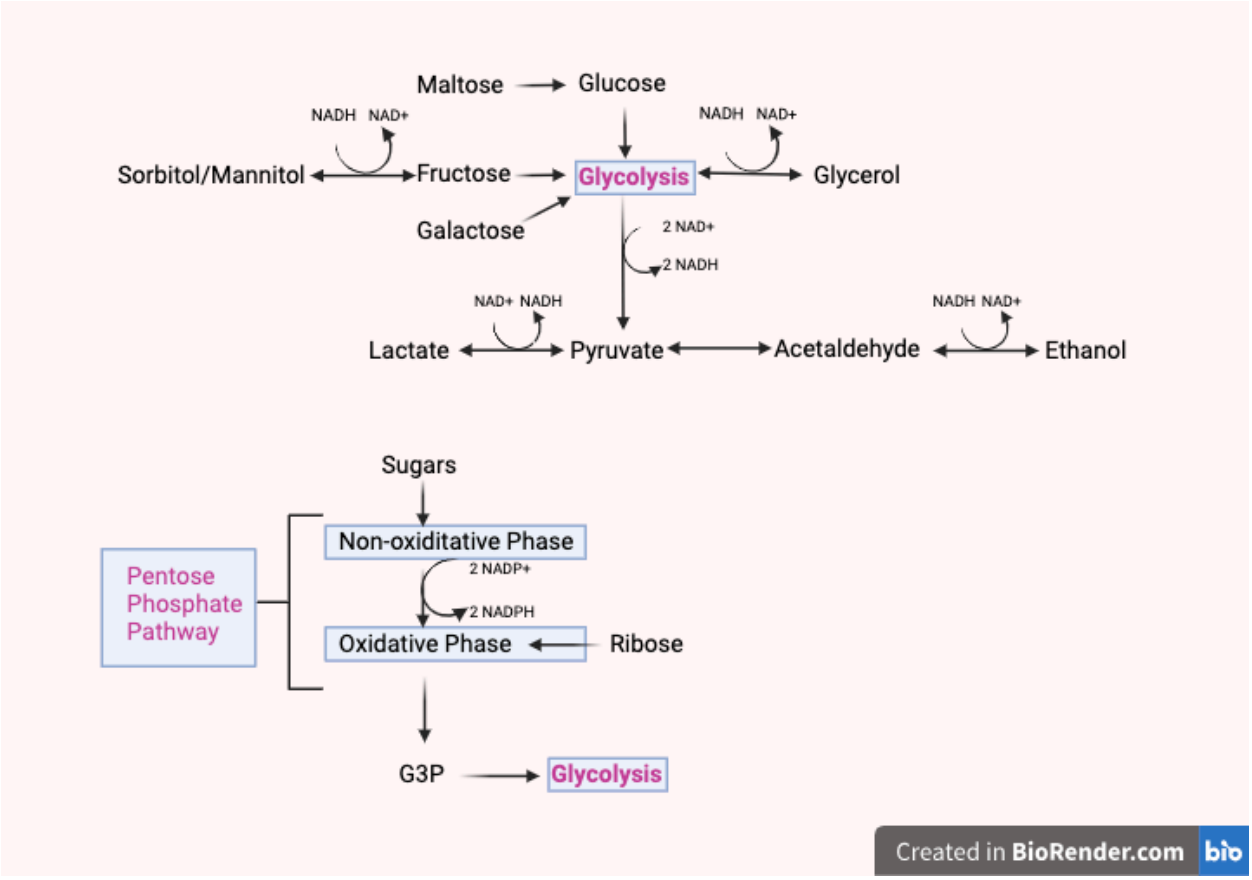

Figure 9. Illustrates the central metabolic pathways for the ten different co-substrates tested, encompassing glycolysis and the pentose phosphate pathway (PPP), without detailing the individual steps. This metabolic map highlights the potential routes these sugars might follow in *S. cerevisiae* and where NADH/NAD+ is used or produced. Notably, under oxidative stress, which can be induced by vanillin, the yeast cells may preferentially channel sugars into the PPP instead of glycolysis. This diversion is significant as the PPP plays a crucial role in managing oxidative stress by generating NADPH, an essential cofactor in cellular redox balancing.

In the screening, cultivation in minimal media was initially conducted using primarily sugars, glycerol, and ethanol as carbon sources together with vanillin at an initial cell optical density (OD620nm) of 0.1. The objective was to compare growth (Figure 10) and measure the produced levels of vanillyl amine, vanillic acid and vanillyl alcohol and the consumption of vanillin (Figure 11). The growth pattern of the yeast differed significantly across various co-substrates (Figure 10). There was also a clear correlation between growth and the consumption of vanillin, with mannose, sucrose, glucose and galactose giving the highest growth and vanillin consumption (Figure 10-11). Ethanol, glycerol, sorbitol, and ribose cultures exhibited minimal growth. Overall, vanillin consumption remained notably low for nearly all co-substrates (less than 1 mM out of 6 mM).

The rationale for using the same weight concentration (10 g/L) of each co-substrate, rather than normalizing based on carbon content, was to streamline the experimental design and to avoid the additional complexity of calculating and adjusting for the varying carbon densities of each co-substrate. This approach was deemed sufficient for an initial screening aimed at observing general growth patterns and vanillin bioconversion profiles. However, this decision may have

influenced the observed growth differences. Given that substrates like glucose and raffinose have different carbon contents per gram, the equal weight concentration does not translate to equal carbon availability. This can significantly affect yeast metabolism, as a higher carbon availability typically supports greater biomass production and could also enhance vanillin uptake and conversion. In light of this, the varied growth responses and vanillin consumption rates across the co-substrates could be partially attributed to the differences in the amount of utilizable carbon provided to the yeast. Future work could involve adjusting the concentration of co-substrates to equalize the available carbon, which would allow for a more controlled comparison of their effects on yeast growth and metabolism.

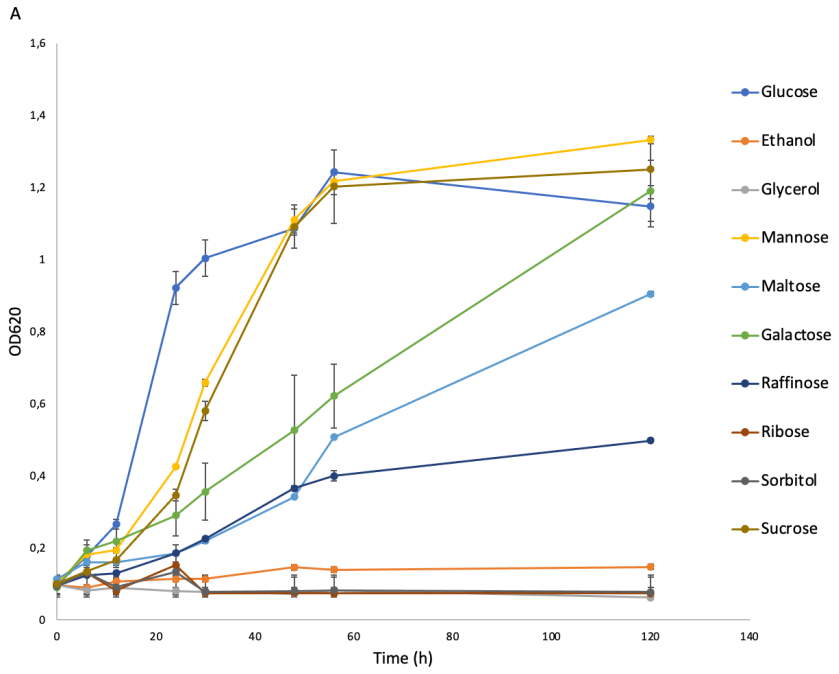

Figure 10. Growth with different co-substrates for an initial OD of 0.1 for strain TMBNM033. The yeast was grown in ~70 mL serum vials in 20 mL YPD media containing vanillin (6 mM) and co-substrate (10 g/L) at 30°C and 180 rpm in a shake incubator.

Glucose, mannose, galactose, maltose and sucrose exhibited the highest production of vanillyl amine and vanillyl alcohol, suggesting their effectiveness as carbon sources for vanilloid production (Figure 11). Ethanol and ribose yielded relatively lower quantities of vanillyl amine, with ethanol additionally leading to some vanillyl alcohol production. Raffinose was the only co-substrate that produced vanillic acid. Glycerol and sorbitol failed to yield any significant products, likely due to the complete absence of cell growth when these co-substrates were utilized. It is worth highlighting that the growth observed with all co-substrates was minimal, suggesting that although they supported some vanilloid synthesis, their overall potential for biomass production in this context was limited.

The minimal growth and vanilloid production observed in *S. cerevisiae* when utilizing glycerol and sorbitol can be attributed to the metabolic characteristics of these substrates. Glycerol requires conversion into glycolytic intermediates, a process that is energetically less favorable compared to the direct metabolism of sugars. This could explain the limited growth seen with glycerol as a co-substrate (Klein et al., 2017). Similarly, sorbitol, not being a preferred carbon source, demands specific transporters and enzymes for metabolism, leading to reduced efficiency in its utilization by yeast. This aligns with the observed minimal growth and vanilloid production with sorbitol (van Aalst, Mans and Pronk, 2022).

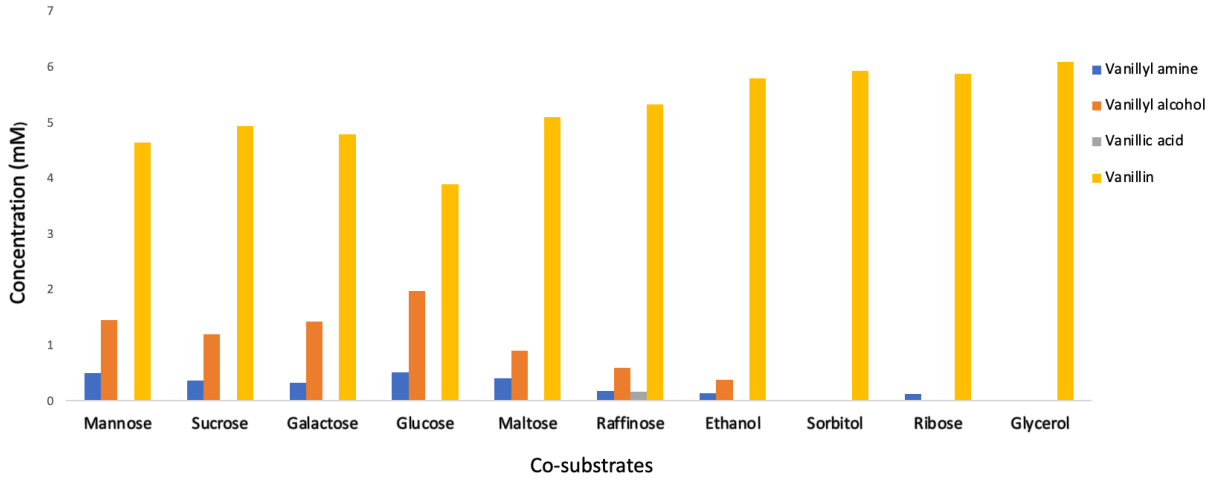

Figure 11. Amount of vanillyl amine, vanillyl alcohol, and vanillic acid produced, and vanillin consumed (start 6 mM) for different co-substrates after 120 hours and a start OD of 0.1 for strain TMBNM033. Sorted by growth. Quantification was done with HPLC with vanillyl amine, vanillyl alcohol, vanillic acid, and vanillin as standards. One replicate was analyzed.

Due to the poor growth observed above, a subsequent experiment was undertaken with an initial OD set at 5, as depicted in Figure 12. In this experiment, seven other co-substrates, 2,3-butanediol, formic acid, glutamic acid, xylose, lactate, mannitol, and rhamnose, known for their limited yeast growth were also evaluated for their production of vanillyl amine and vanillic acid. This time, an increase in vanillyl amine, vanillic acid, and vanillyl alcohol production was observed for the co-substrates that had previously been tested with growing cells, at an initial OD of 0.1. Additionally, there was a noticeable rise in vanillin consumption across these co-substrates. Notably, co-substrates such as ethanol, glycerol, and sorbitol, which had previously exhibited minimal production, displayed significantly enhanced yields. Furthermore, all co-substrates that had previously shown no detectable vanillic acid production, were able to produce this compound in resting cells assays. Among the co-substrates investigated, certain compounds, such as formic acid and glutamic acid, demonstrated negligible production of these target products in comparison to the control. Since the amount of vanillin used differed significantly between the co-substrates, a detailed discussion of the product yield is also presented below.

The observation of vanillyl alcohol production in the *ADH6*-deleted strain of *S. cerevisiae* presents an intriguing metabolic scenario. Typically, *ADH6* is implicated in the reduction of aldehydes to alcohols. The deletion of *ADH6* alters the yeast's metabolic network, potentially leading to a rerouting of metabolic pathways. This rerouting may increase the flux through alternative enzymatic routes that favor the production of vanillyl alcohol. The absence of *ADH6* could result in the accumulation of intermediates that are then processed by other alcohol dehydrogenase, for example *ADH7*, culminating in vanillyl alcohol synthesis (Nguyen et al, 2015).

Moreover, the presence of metabolic products in the control strain underscores the inherent metabolic versatility of *S. cerevisiae*. Even in the absence of co-substrate yeast cells naturally engage in a diverse array of metabolic activities, producing vanillyl amine, vanillic acid, and vanillyl alcohol from vanillin. This baseline metabolic activity is crucial for maintaining cellular functions and can result in the formation of small amounts of metabolic products under standard growth conditions. It serves as an important control in experimental setups, indicating the yeast's native metabolic state against which the effects of co-substrate addition can be compared.

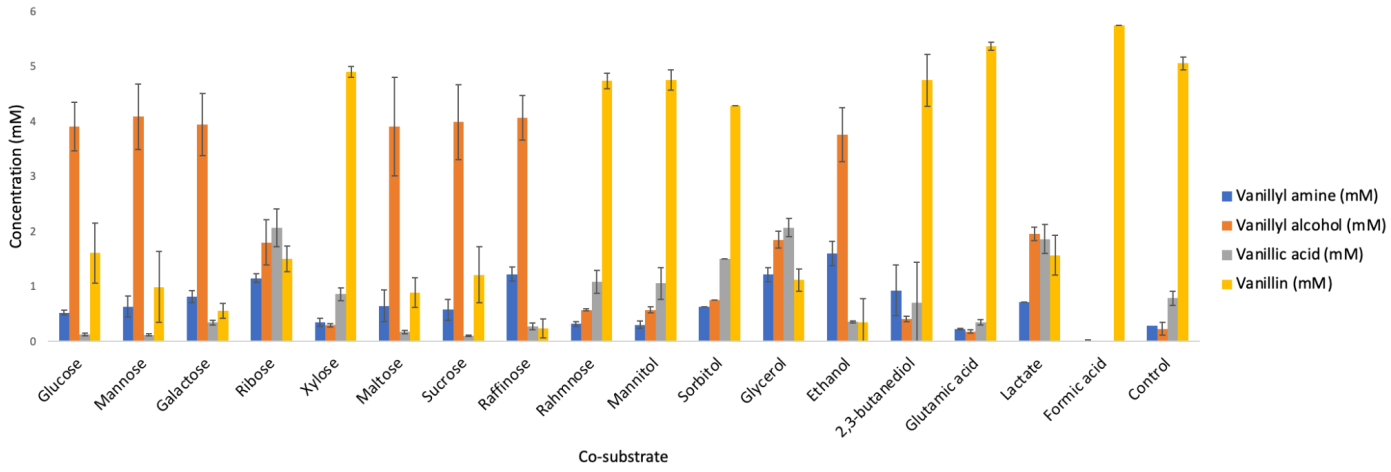

Figure 12. Amount of vanillyl amine, vanillyl alcohol, and vanillic acid produced, and vanillin consumed (start 6mM) for different co-substrates after 48 hours and a start OD of 5 for strain TMBNM033. Quantification was done with HPLC with vanillyl amine, vanillyl alcohol, vanillic acid, and vanillin standard. Two replicates were analyzed, except for sorbitol which only had one replicate.

3.2.1 Vanillyl amine production yield

In the vanillin bioconversion assays, the co-substrate 2,3-butanediol had the highest vanillyl amine yield (Table 3). However, it is noteworthy that the first replicate displayed an unexpectedly high yield, exceeding 1. This result implies that more vanillyl amine was produced than the vanillin was consumed, which is not in accordance with the expected stoichiometry of the reaction. Furthermore, the second replicate encountered problems with agitation in the water bath overnight, potentially affecting the yeast cultivation. Therefore, new replicates would be required to confirm the results for this co-substrate

Sorbitol had the second highest vanillyl amine yield; however, the amount produced was fairly low since the vanillin consumption was low. Also, one replicate from sorbitol was lost in the process, making the data less reliable. Glutamic acid emerged as the third-best co-substrate in terms of vanillyl amine yield (Table 3). However, the amount of vanillyl amine produced was significantly lower compared to other co-substrates and the control (without co-substrate). This suggests that while glutamic acid did contribute to a higher vanillyl amine yield, it is not the most efficient co-substrate for this particular bioconversion.

Ethanol had the highest vanillyl amine yield of 0.29 and also displayed a unique characteristic with the highest relative ratio, indicating that it produced a substantial amount of vanillyl amine in comparison to vanillic acid and vanillyl alcohol (Table 3). However, it is important to note that ethanol also generated a considerable amount of vanillyl alcohol. Ethanol is initially degraded to acetaldehyde through enzymatic reactions, a process that involves the conversion of NAD+ to NADH. Additionally, acetaldehyde is further metabolized to acetate, again utilizing NADP+ while producing NADPH. This surplus of NAD(P)H generated during ethanol metabolism might play a crucial role in facilitating the production of vanillyl amine (de Smidt et al, 2008). Ethanol also had a high conversion of vanillin, which might have contributed to the higher vanillyl amine yield due to more pyruvate entering the TCA cycle, producing more NADH (Nolfi-Donegan et al, 2020).

Table 3. Vanillyl amine and vanillic acid yields (mol vanillyl amine/mol consumed vanillin) and relative ratio of vanillyl amine and vanillic acid (mol vanillyl amine/(mol vanillic acid + vanillyl alcohol)) for the co-substrates and control without co-substrate for strain TMBNM033 after 48 h. VA, vanillyl amine. VAC, vanillic acid. VAA, vanillyl alcohol, two replicates were analyzed except for sorbitol due to error in the handling of the sample.

Co-Substrate	Type	VA concentration (mM)	VAA concentration (mM)	VAC concentration (mM)	VA Yield	VAC Yield	VAC Relative ratio	VA Relative ratio
Glucose	Monosaccharide	0.52 ± 0.04	3.76 ± 0.4	0.13 ± 0.03	0.12 ± 0.005	0.030 ± 0.01	0.03 ± 0.01	0.13 ± 0.003
Mannose	Monosaccharide	0.63 ± 0.2	4.09 ± 0.6	0.12 ± 0.02	0.12 ± 0.02	0.023 ± 0.01	0.03 ± 0.0001	0.15 ± 0.02
Galactose	Monosaccharide	0.82 ± 0.2	3.95 ± 0.7	0.35 ± 0.04	0.14 ± 0.02	0.061 ± 0.01	0.07 ± 0.001	0.16 ± 0.04
Ribose	Monosaccharide	1.15 ± 0.1	1.80 ± 0.4	2.07 ± 0.3	0.25 ± 0.005	0.45 ± 0.001	0.70 ± 0.003	0.30 ± 0.04
Xylose	Monosaccharide	0.35 ± 0.1	0.3 ± 0.03	0.86 ± 0.1	0.26 ± 0.07	0.65 ± 0.03	1.36 ± 0.0	0.26 ± 0.03
Maltose	Disaccharide	0.64 ± 0.3	3.91 ± 0.9	0.17 ± 0.03	0.12 ± 0.06	0.032 ± 0.01	0.04 ± 0.003	0.15 ± 0.04
Sucrose	Disaccharide	0.58 ± 0.2	3.99 ± 0.7	0.10 ± 0.02	0.12 ± 0.03	0.022 ± 0.01	0.02 ± 0.023	0.14 ± 0.02
Raffinose	Oligosaccharide	1.22 ± 0.1	4.07 ± 0.4	0.28 ± 0.06	0.21 ± 0.02	0.047 ± 0.05	0.05 ± 0.02	0.28 ± 0.008
Rhamnose	Deoxy sugar	0.32 ± 0.04	0.58 ± 0.01	1.08 ± 0.2	0.21 ± 0.01	0.72 ± 0.07	1.2 ± 0.003	0.19 ± 0.003
Mannitol	Sugar alcohol	0.31 ± 0.06	0.58 ± 0.1	1.06 ± 0.3	0.21 ± 0.02	0.72 ± 0.1	1.2 ± 0.0003	0.19 ± 0.0003

Sorbitol	Sugar alcohol	0.64	0.76	1.50	0.35	0.82	0.70	0.28
Glycerol	Sugar alcohol	1.22 ± 0.1	1.85 ± 0.2	2.07 ± 0.2	0.25 ± 0.04	0.42 ± 0.02	0.68 ± 0.1	0.31 ± 0.03
Ethanol	Alcohol	1.60 ± 0.2	3.76 ± 0.5	0.36 ± 0.02	0.29 ± 0.02	0.065 ± 0.01	0.07 ± 0.01	0.39 ± 0.0010
2,3-butanediol	Glycol	0.93 ± 0.4	0.41 ± 0.1	0.70 ± 0.7	0.77 ± 0.6	0.44 ± 0.4	0.64 ± 0.7	1.30 ± 1.3
Glutamic acid	Amino acid	0.22 ± 0.02	0.18 ± 0.03	0.35 ± 0.1	0.33 ± 0.06	0.50 ± 0.01	0.87 ± 0.2	0.42 ± 0.04
Lactate	Carboxylic acid	0.72 ± 0.1	1.96 ± 0.1	1.87 ± 0.3	0.15 ± 0.01	0.39 ± 0.03	0.49 ± 0.2	0.19 ± 0.002
Formic acid	Carboxylic acid	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Control	-	0.28 ± 0.03	0.23 ± 0.1	0.79 ± 0.1	0.21 ± 0.01	0.59 ± 0.05	1.55 ± 0.2	0.28 ± 0.04

3.2.2 Vanillic acid production yield

For applications where the objective is to maximize vanillic acid production or maintain an equivalent ratio of vanillic acid to vanillyl amine, it becomes crucial to select a co-substrate that also contributes to the generation of NAD+, the co-factor utilized by vanillin dehydrogenases in the conversion of vanillin into vanillic acid.

NAD+ plays a crucial role in cellular metabolism by acting as an electron carrier. It is primarily regenerated within the cells when NADH is oxidized back to NAD+ in the electron transport chain (ETC) during aerobic respiration (Nolfi-Donegan et al, 2020). However, in oxygen-limited or anaerobic conditions, the electron transport chain becomes inactive. Under such circumstances, there is a reduced availability of molecular oxygen (O2), which serves as the terminal electron acceptor in the ETC, as a result disrupting the normal flow of electrons and, consequently, the regeneration of NAD+ (Nolfi-Donegan et al, 2020). Consequently, under oxygen-limited conditions, cells, in the presence of glucose or other compounds that can be converted into pyruvate, will resort to fermentation as an alternative metabolic pathway. During fermentation, pyruvate plays a crucial role in the regeneration of NAD+ from NADH, a vital requirement for sustaining glycolysis and various metabolic processes. This occurs through the conversion of pyruvate to acetaldehyde, followed by the NADH-dependent reduction of acetaldehyde to ethanol (Willis 1990). Therefore, the low amount of vanillic acid produced for most of the saccharides is likely due to the low amount of NAD+ being produced due to the electron transport chain being inactive. The NAD+ produced from the conversion of pyruvate to ethanol might instead go to glycolysis as it is a vital pathway for the cell.

Among the co-substrates tested in the present experiment, glycerol, ribose, and lactate had the highest vanillic acid yield, and had an overall more balanced production of vanilloids. Glycerol can be metabolized to glucose through gluconeogenesis and enter the Pentose Phosphate Pathway (PPP) or enter the glycolysis. In glycolysis, glycerol is converted to dihydroxyacetone

phosphate (DHAP), producing NADH in the process. In the PPP, glycerol is transformed into glucose-6-phosphate (G6P), leading to the production of NADPH. Subsequently, the pyruvate formed from these pathways can undergo fermentation to yield ethanol, which generates NAD+ (Xiberras et al, 2019). This observation aligns with the fact that yeast utilizing glycerol as a co-substrate yielded a relatively equal amount of vanillyl amine, vanillic acid, and vanillyl alcohol. Ribose on the other hand, is only metabolized in the non-oxidative phase in the PPP (Bertels et al, 2021). This means that no NADPH is formed, which aligns with the lower amount of vanillyl alcohol comparable to other sugars that enters the PPP at the oxidative phase.

4. Discussion

Regarding the BGA biosynthesis by the strains TMBNM020-TMBNM031, no product was detected in HPLC analysis. However, it is not clear whether this is because the strains were unable to convert vanillyl amine and vanillic acid to BGA or whether the detection method utilized was unable to detect BGA. Previously, Muratovska and collaborators (2022) obtained the highest concentration of nonivamide (10.6 mg/L) using the strain TMBNM025, suggesting that the maximal concentrations that can be obtained of BGA might be relatively low and therefore, challenging to be detected using HPLC analysis.

The HPLC analysis was also utilized to monitor any changes in the levels of vanillyl amine and vanillic acid, as this could indicate their utilization in the synthesis of BGA. There was a small reduction in concentration of vanillyl amine in most strains. For most strains an increase in vanillic acid was also observed, indicating that the method used was not perfect and that there was no bioconversion and very little metabolic activity going on.

The best performing strain in terms of vanillyl amine consumption were TMBNM025 (IpfF/CaAT), which also were the best performing strain in nonivamide production (Muratovska et al, 2022). The HPLC analysis indicated a decline in vanillyl amine from 6.6 mM to approximately 4.2 mM between 0h and 120 h, and an increase in vanillic acid from 5.8 mM to 6.3 mM; however, no new detectable peaks emerged in the HPLC chromatogram. Due to the increase of vanillic acid no BGA was likely formed, as the conversion of vanillyl amine and vanillic acid should be stoichiometrically converted into BGA (Figure 1), and thus a simultaneous decrease in both of these compounds should be observed.

The control also had a reduction in vanillyl amine and vanillic acid from 6.5-5.5 mM. This could be attributed to the binding of these compounds to the yeast cells resulting from adsorption, or some other type of molecular interaction where the compounds become physically associated

with the yeast cells without necessarily undergoing metabolic conversion. The adsorption can result in a decrease in the concentration of these compounds in the solution because they are effectively removed from the supernatant and attached to the yeast cells. Only one replicate was made in CBS-media while two replicates were made in YPD-media. When the strains grown in YPD-media were analyzed in HPLC no new detectable peaks were observed. The problem with YPD-media however, was that the consumption of vanillyl amine could not be calculated due vanillyl amine having the same retention time as a component of the YPD-media.

One potential explanation why no BGA synthesis occurred might be due to the CL enzymes not recognizing vanillic acid, as this aspect had not been tested previously. NAT enzymes have been shown to recognize vanillyl amine (Muratovska et al 2022). Moreover, our experimental findings provide evidence supporting the inert nature of vanillyl amine and vanillic acid within yeast. This observation strongly suggests the absence of any discernible side reactions involving these compounds. The significance of this lies in the potential identification of a viable amide-forming cascade within yeast. If such a cascade can be successfully identified, it shows that yeast is a good fit for these reactions. It also suggests that yeast could become a strong and flexible platform for future uses in making amides and other related biochemical processes.

In the bioconversion of vanillin into vanillyl amine, vanillic acid and vanillyl alcohol, the choice of co-substrate plays a pivotal role in determining the main product to be formed. This is because different co-substrate can affect the redox balance in the cells, affecting directly the reactions related to vanillin bioconversion: vanillin reductases rely mainly on NADPH; vanillin dehydrogenases rely on NAD+, and alanine dehydrogenase from *Bacillus subtilis* (BsAlaDH), which enables an efficient reductive amination of vanillin, is NADH-dependent. Hence, the selection of a co-substrate depends on the desired product, since the metabolism of each co-substrate leads to different ratios of NAD+/NADH and NADP+/NADPH in the cell. Another important factor to consider is that an accumulation of pyruvate can inhibit the production of vanillyl amine as it is a strong inhibitor of the amine transaminase CvTA (Muratovska and Carlquist, 2023). The focus of this study was to maximize yields of vanillyl amine and vanillic acid and to preferably find a co-substrate that produces equal ratios of each product.

A clear trend could be seen where all monosaccharides, disaccharides, and oligosaccharides, except xylose and ribose, were found to produce an equal amount of vanillyl amine, vanillic acid, and vanillyl amine. The vanillyl amine yield was about 0.12 for all except for raffinose with the highest yield of 0.21. For all of them vanillic acid yield was very low (below 0.1). However, they all produced substantial amounts of vanillyl alcohol. This can likely be attributed to these co-substrates entering the pentose phosphate pathway, which is known for its capacity to generate NADPH. The pentose phosphate pathway serves a critical role in protecting yeast cells from oxidative stress. NADPH, a product of this pathway, is an indispensable cofactor for enzymes involved in the defense mechanisms against oxidative damage (Slekar et al, 1996). The

presence of vanillin has been shown to induce an oxidative stress response in *S. cerevisiae* (Nguyen et al, 2014). Consequently, the activation of the pentose phosphate pathway and the increased production of NADPH can help counteract this oxidative stress and support the conversion of vanillin to vanillyl alcohol. Ribose on the other hand does not enter the non-oxidative phase of the PPP and thus does not produce as much NADPH, which might be the reason for the lower amount of vanillyl alcohol produced. Xylose cannot be assimilated by *S. cerevisiae* which is probably the cause why it produced an equal amount of vanillyl amine, vanillic acid, and vanillyl alcohol as the control experiment lacking co-substrate.

Glycerol, ribose, and lactate had a much higher vanillic acid yield comparatively to the other co-substrates. Overall, the amount of vanillyl amine, vanillic acid, and vanillyl alcohol produced were more balanced for these co-substrates meaning that the redox homeostasis inside the yeast cells were more balanced when using these co-substrates. Ethanol, sorbitol, and glutamic acid had the highest yield of vanillyl amine; however, the amounts glutamic acid produced were significantly lower than the control. Ethanol had a higher relative ratio of vanillyl amine than sorbitol, meaning that ethanol produced a higher percentage of vanillyl amine than vanillic acid and vanillyl alcohol compared to sorbitol, whereas sorbitol production was more balanced. Glycerol and ribose had a relatively high yield of both vanillyl amine and vanillic acid which is advantageous when aiming for an equal production of vanillyl amine and vanillic acid.

As previously mentioned, many co-substrates produced a significant amount of vanillyl alcohol, even in the absence of the *ADH6* gene, which is the dominant gene responsible for the conversion of vanillin to vanillyl alcohol (Muratovska and Carlquist, 2023). This indicates that other alcohol dehydrogenases also catalyze this reaction. Among the co-substrates tested, glucose and ethanol exhibited lower vanillyl amine yields compared to a previous study by Muratovska et al (2023). However, this could be attributed to different experimental setups and the supplementation of alanine in the media. In order to produce less vanillyl alcohol and to enhance the yield of vanillyl amine and vanillic acid, it is recommended to consider the deletion of the NADPH-dependent alcohol dehydrogenase 7 (*ADH7*) gene. This genetic alteration is likely to be the root cause of the continued production of vanillyl alcohol, despite the deletion of the *ADH6* gene (Nguyen et al, 2015).

5. Conclusion

No enzyme combinations that we tested resulted in significant BGA production, as indicated by the absence of conversion of vanillyl amine and vanillic acid. In a BGA-producing reaction, we would anticipate a simultaneous decrease in both vanillyl amine and vanillic acid. As seen in Figure 2, 1 molecule of vanillic acid and 1 molecule of vanillyl amine is consumed in this

reaction. However, this was not observed. Instead, only minor variations were noted within the range of error in the HPLC method, suggesting that no significant conversion occurred.

In our screening of various co-substrates, it was evident that choice of co-substrate had a high impact on the production of vanillyl amine, vanillic acid, and vanillyl alcohol. There was a clear trend where monosaccharides, disaccharides, and oligosaccharides produced a lot of vanillyl alcohol likely due to their NADPH generation via the pentose phosphate pathway. Glycerol, ribose, and lactate produced a higher yield of vanillic acid, and an overall more balanced production of vanilloids. Ethanol emerged as the most effective co-substrates for vanillyl amine production, in terms of yield.

6. Popular Scientific Summary

Plastics, derived from fossil fuels, pose significant environmental challenges, fueling a quest for sustainable alternatives. In this study, vanillin, a phenolic compound derived from lignin which is an abundant and renewable natural polymer that is present in wood and other agricultural crops, was explored as a potential eco-friendly substitute for traditional industrial chemicals. The focus was on microbial production of chemical building blocks from vanillin using genetically engineered yeast, tiny organisms commonly used in baking and brewing, but genetically modified to convert vanillin into other chemicals. This process involves amide-forming enzymes, which are specialized tools within the yeast, allowing it to construct complex molecules. The study also investigated the influence of adding different co-substrates, additional natural ingredients, to see how they influence this transformation and to optimize the production of vanilloids, compounds derived from vanillin.

Despite promising prospects, the study encountered challenges in the biosynthesis of chemical building blocks from vanillyl amine and vanillic acid. Specifically, the strains showed no detectable product in the analysis, raising questions about their capability or the methodology used. The study suggested that the inert nature of vanillyl amine and vanillic acid within yeast indicated the absence of discernible side reactions, making yeast a potential platform for future amide-forming reactions.

In the investigation of co-substrates effects on vanilloid production, we saw that the choice of co-substrate played a crucial role in determining the main product formed during the bioconversion of vanillin. Different co-substrates affected the redox balance within cells, which are chemical reactions involving the transfer of electrons, crucial for the yeast to change vanillin into various products. This in turn influences the type and quantity of vanilloids produced. Notably, monosaccharides, disaccharides, and oligosaccharides led to vanillyl alcohol production, while glycerol, ribose, and lactate led to a more balanced production of vanilloids.

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