Hydrocortisone Production Through the Fermentation of *Saccharomyces cerevisiae*

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Course: CHBE 221

Date: April 8th, 2025

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1.0 Abstract

Hydrocortisone is an anti-inflammatory steroid that is in high demand for medical use. It is a steroid hormone which is extensively used to treat autoimmune diseases and inflammation and is produced in various medicinal forms due to rising clinical demand [1]. This project synthesizes hydrocortisone using genetically modified Saccharomyces cerevisiae yeast via fermentation. Optimal yeast growth and efficient hydrocortisone production occur in a chemostat continuous stirred-tank reactor (CSTR), which allows precise fermentation condition control [2], [3]. The reactor operates at 25°C with minimal light exposure, maintains a stable pH of 6.5 using citrate-phosphate buffering, and utilizes glucose, ammonium sulfate, essential vitamins, and trace elements as growth media [4], [5], [6]. Effective mixing is achieved through a Rushton radial flow impeller, while automated sensors ensure nutrient stability, dissolved oxygen control, and reactor volume management [6].

Since *S. cerevisiae* does not naturally synthesize hydrocortisone, genetic modifications include introducing CYP17A1 and CYP21A1 genes, encoding cytochrome P450 enzymes crucial for glucose-to-hydrocortisone conversion [4]. The codon sequences of these genes will be optimized for *S. cerevisiae.* The TEF1 promoter initiates transcription [7], PRY proteins facilitate hydrocortisone secretion [8], and the Tsynth27 terminator ensures mRNA stability [9].

The hydrocortisone product is designed as a water-soluble powder, intended for dilution by medical practitioners immediately before use [10]. Meeting pharmaceutical standards, the product achieves at least 98% purity to comply with US Pharmacopeia regulations [11], [12], [13]. The continuous process outlined begins with an AKW hydrocyclone, effectively separating yeast strains from fermentation broth through centrifugal force [14] . Subsequent thermal precipitation in a continuous decanter initiates crude hydrocortisone separation [15], [16]. Further purification utilizes high-performance liquid chromatography (HPLC) with reversed-phase ODS-silica columns, efficiently isolating hydrocortisone [17]. Final crystallization involves dissolving hydrocortisone in propylene glycol at elevated temperatures, followed by cooling-induced crystallization, rinsing, and recrystallization to exceed 98% purity [18], [19], [20], [21]. The purified hydrocortisone undergoes vacuum drying at 40°C, resulting in a high-purity, medical-grade powder suitable for clinical applications [22].

2.0 Bioproduct and Cell Selection

2.1 Bioproduct Selection

Hydrocortisone is the selected bioproduct. Hydrocortisone, also known as 11β,17α,21-trihydroxypregn-4-ene-3,20-dione, is a corticosteroid hormone or glucocorticoid naturally produced in the adrenal gland [23]. See Figure 1 for the molecular structure of hydrocortisone. Cortisone is a hormone involved in the body's response to stress/anxiety, however it is also used as a medicinal steroid to calm down immune responses to pain [24].

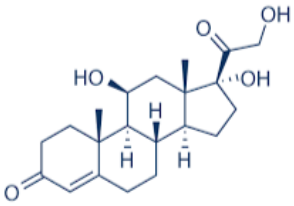

Figure 1: Hydrocortisone molecular structure [3]

Hydrocortisone comes in various forms (injection shot, creams, ointments, etc.) and is commonly administered by physicians as an anti-inflammatory [23]. Hydrocortisone is an ideal compound for the bio-production project due to its broad medicinal applications and a manufacturing process optimized for bio-based production. Bio-production greatly reduces the production steps when compared to the chemical synthesis pathway for producing hydrocortisone which has 8 intermediate steps for being synthesized from dextropregnenolone [25].

2.1.1 Applications and Uses of Hydrocortisone

Hydrocortisone is an anti-inflammatory steroid available in multiple forms. Skin conditions like eczema, contact dermatitis, heat rash, insect bite reactions, and psoriasis are treated with hydrocortisone creams, ointments, or lotions [24]. Hemorrhoids are treated with hydrocortisone as a suppository, cream, ointment, or spray. To add, mouth ulcers are relieved with hydrocortisone buccal tablets, which adhere to the mouth's interior and dissolve to release hydrocortisone medication [24]. Joint pain, including tennis elbow, painful tendons, and bursitis, is managed with hydrocortisone injections [24]. Additionally, adrenal gland conditions such as Addison's disease or hypopituitarism are treated with hydrocortisone tablets, also prescribed for individuals who have had their adrenal glands removed [24]. Hydrocortisone's glucocorticoid receptor and annexin A1 antagonism are responsible for its anti-inflammatory properties.

Downstream effects of the drug inhibit inflammatory transcription factors including phospholipase A2 and NF-kappa B. It also promotes anti-inflammatory transcription factors such as interleukin [26].

The short-term effects decrease vasodilation, capillary permeability, and leukocyte migration to sites of inflammation [27]. Low doses are anti-inflammatory while high doses are immunosuppressive. Extended, high dosage use leads to glucocorticoids binding to mineralocorticoid receptors, increasing sodium and decreasing potassium levels. Chronic use may also cause significant adverse effects such as glaucoma, hypertension, osteoporosis, psychosis, leukemia, and schizophrenia [27]. Repeated exposure may cause damage to fertility and specific organs [28].

Hydrocortisone is a valuable pharmaceutical compound due to its widespread use in treating inflammatory and autoimmune conditions. Patients, healthcare providers, and pharmaceutical companies rely on hydrocortisone for its effectiveness at alleviating symptoms and improving patient's quality of life. Producing hydrocortisone at a large scale is crucial to meeting the growing global demand for anti-inflammatory medications.

2.1.2 Market information

Inflammatory autoimmune diseases are becoming more common, driving growth in the hydrocortisone market. Increased demand for hydrocortisone is expected to sustain this growth trend. Hydrocortisone is already produced on a large scale by major companies including Pfizer Inc., Johnson & Johnson, etc... The market grew from $1.52 billion in 2024 to $1.62 billion in 2025, with North America being the largest contributing region [13]. The hydrocortisone market has a compound annual growth rate (CAGR) of 6.7% [23]. Figure 2 provides a graphical representation of the 2025 market report, illustrating the projected increased market value for 2026–2029 [23]. It is difficult to estimate the global quantities of hydrocortisone required however, it could be estimated that an average sized hospital uses 3 to 4 grams per day [8]. Though this does not include many other users of hydrocortisone, it can be concluded that worldwide a large quantity is required [29], [30].

Figure 2: Graph of the Hydrocortisone Global Market Report in 2025 [23]

2.1.3 Production Requirements

Hydrocortisone is naturally produced in the adrenal complex of mammals [4]. Through recombinant DNA, bacteria such as *Escherichia coli* and fungi including *Saccharoymces cerevisiae*, have all been used for production [31], [32]. Bacteria and fungi have increased efficiency due to their simplicity and higher cell concentrations [33]. Yeasts like *S. cerevisiae* are preferred over bacteria, because they are eukaryotic, capable of conducting more complex multi-step enzymatic reactions, such as the one required to produce hydrocortisone [34]. Media supporting yeast growth requires sugars, amino acids, vitamins, minerals, and oxygen [35].

2.1.4 Physical and Chemical Properties of Hydrocortisone

Hydrocortisone is a corticosteroid which acts on glucocorticoid receptors. It weighs 362.5g/mol and has a melting point of 220°C. It is sparingly soluble in most solvents, with a solubility of 0.32mg/ml and 15mg/ml in water and ethanol respectively [23]. This low solubility can be used in separation processes.

The stability of the compound depends on its form. Manufacturers recommend tablets be consumed within 36 months while oral solutions prepared from tablets last for 30 days with proper storage [26], [28]. Once ingested, hydrocortisone is eliminated from the blood within 6 hours while effects last for 8-12 hours [27].

2.2 Cell Selection

To produce hydrocortisone, *S. cerevisiae* (also known as Baker's yeast), a unicellular eukaryote and species of yeast, has been chosen. It is routinely used as a model in biological research and

its genome is completely sequenced, which is beneficial when genetically engineering for biosynthetic processes [4].

2.2.1 Cell Justification and Hydrocortisone Productions

The *S. cerevisiae* cells are not capable of producing hydrocortisone naturally, yeasts have no capacity for steroid biosynthesis. However, genetically modified *S. cerevisiae*, has been developed to produce hydrocortisone in a single fermentation step from simple carbon sources. It has been engineered to contain mammalian cytochrome P450 enzymes that initiate hydroxylation reactions, along with 21-hydroxylase and 11β-hydroxylase, which facilitate the conversion of hydrocortisone precursors. The process produces 11-deoxycortisol and corticosterone as the only by-products in ideal conditions [4].

The *S. cerevisiae* cell has advantages over alternatives such as bacteria and mammalian cells. For example, yeasts naturally produce sterols, a compound making them good hosts for steroid hormone pathways [4]. These native sterol biosynthesis pathways reduce the amount of genetic engineering required and provide a compatible metabolic environment for efficient hydrocortisone production. In addition, yeast can handle post-translational modifications, membrane-bound enzymes, and sterol processing, all of which are required for the synthesis of hydrocortisone [36]. Compared to mammalian cells, yeast has a faster growth rate and is more cost-effective [4]. The eukaryotic nature of the yeast makes it more suitable for steroid biosynthesis, compared to prokaryotic bacteria, since it has mitochondria and endoplasmic reticulum. These organelles enable the proper localization and function of membrane-bound enzymes required for the complex oxidative reactions in steroid biosynthesis, such as cytochrome P450s, which bacteria are not equipped to handle as efficiently [34]. It is also more stable, more scalable for larger production, and it avoids some ethical/regulatory issues that arise when using animal-derived products (mammalian cells) [36]. Our group has selected the S. cerevisiae cell to produce hydrocortisone due to these benefits.

2.2.2 Nutritional Classification

The *S. cerevisiae* cells are classified as facultative anaerobic chemoorganoheterotrophs. This means they derive energy and carbon from organic compounds. Glucose is the organic compound used in hydrocortisone production as the substrate. It has been chosen over other simple sugars, due to its high availability and the fact that it is inexpensive. *S. cerevisiae* cells

can operate with or without an oxygen supply, however they strongly prefer to operate with oxygen for optimal energy production [5]. For our process we need oxygen as the bio-synthesis pathway for producing hydrocortisone requires mammalian P450 enzymes that require oxygen [37]. *S. cerevisiae* also requires sources of nitrogen, hydrogen, sulfur and phosphorous, as well as certain amino acids depending on the desired product. More details about the nutrients will be given in section 4.1.2.

2.2.3 Relevant Physical and Biochemical Characteristics

S. cerevisiae cells maintain a moderate growth rate, doubling over 1.5 to 2 hours under optimal conditions. Engineered strains of the yeast may grow slower with reduced growth efficiency, because they can cause metabolic stress affecting their overall growth and productivity [5]. The yeast cells are unicellular and grow optimally at 30°C with a pH range of 4.5-6.5 [38]. There is also the potential risk of the accumulation of intermediate steroid by-products from the yeast, which decreases the yield of the desired hydrocortisone [5].

3.0 Genetic Engineering

3.1 Recombinant Proteins

S. cerevisiae does not naturally produce hydrocortisone; therefore, the yeast strain needs to be genetically modified. A family of mammalian P450 cDNA genes, has been identified as producing enzymes that efficiently convert the glucose substrate into hydrocortisone [4]. Four cytochromes have been identified as necessary for production: cP450 17α-hydroxylase, cP450 21-hydroxylase, cP450 11β-hydroxylase, and P450scc [39], [4], [31], [40], [41]. P450 cDNAs refers to complementary DNA (cDNA) encoding cytochrome P450, a large family of enzymes involved in metabolizing drugs [41]. Outlined below is the expression cassette of the genome that will be inserted into the *S. cerevisiae* cells.

3.2 Expression Cassette

There are genes being focused on that are inserted into the *S. cerevisiae* cells are CYP17A1 and CYP21A1. These genes produce the cytochrome P450 enzymes 17α- and 21- hydroxylase respectively, required by the cell to convert the glucose substrate into hydrocortisone, through a series of biosynthesis steps in the cell shown in Figure 3. The coding regions of each gene will be expressed together using the same promoter and termination sequence to produce both required enzymes. A ribosome binding site will be present in between the genes to express them

as two separate proteins. These genes can be found in most mammals, although they have only been studied and sourced from humans, mice and rats [42].

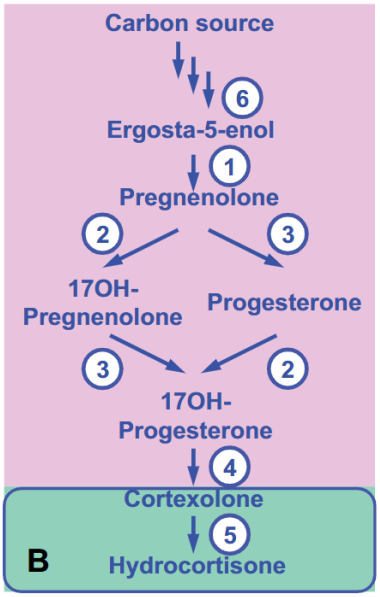

Figure 3: Hydrocortisone steroid biosynthesis using P450 hydroxylase enzymes [4]

The CYP17A1 gene that codes for P450c 17α-hydroxylase, is located on the human chromosome 10q24.3 and consists of 10-exon spanning 3,100 base pairs in length [39]. These 10 exons represent the number of coding regions of a gene that are transcribed into RNA and then translated into the 17α-hydroxylase enzyme, which is crucial for converting pregnenolone into progesterone [4]. See point 3 in Figure 3. The CYP21A1 gene, which codes for P450c 21-hydroxylase, is located on chromosome 6p21.31. It is made up of 8 exons and has a length of 5,700 base pairs [39]. This enzyme converts progesterone into cortexolone (11-deoxycortisol), which is then further converted into hydrocortisone by the cP450 11β-hydroxylase enzyme expressed by the CYP11B1 gene [31]. See points 4 and 5 in Figure 3. Other important genes involved in this pathway include the DHCR7 gene, which encodes an enzyme responsible for removing specific double carbon bonds from the glucose substrate to produce ergosta-5-enol [43]. See point 6 in Figure 3. Then the CYP11A1 gene codes for the cytochrome P450 side-chain cleavage enzyme that converts ergosta-5-enolin to pregnenolone [4], [40]. See point 1 in Figure 3.

A fusion enzyme can optimize production. Fusion enzymes between yeast NADPH–P450 reductase and human hepatic cytochrome P450 3A4 (CYP3A4) have been shown to increase testosterone 6β-hydroxylation activity [44].The fusion enzyme can efficiently transfer electrons from NADPH to CYP3A4, increasing the rate at which the energy driven cytochrome can catalyze the hydroxylation [44]. CYP17A1 and CYP21A1 are similar human cytochromes which require electron transfer from NADPH-P450, indicating that a fusion protein between the

respective cytochromes and NADPH-P450 reductase could increase the enzymatic activity [44]. The electron transfer requirement indicates that the fusion of NADPH-P450 reductase would increase the rate of reaction in the same way observed in the CYP3A4 fusion enzyme. To construct the gene, an adaptation of the method to generate the expression plasmid pAFCR1 for a fusion enzyme between NADPH-P450 reductase and rat cytochrome P450 1A1 can be used [45]. As mentioned above, each cytochrome will be in the same gene as NADPH-P450 reductase to simultaneously translate them into one fusion enzyme. See Appendix A for genomic sequence of NADPH-P450 reductase.

In *S. cerevisiae*, typical protein signaling via α-mating factor secretion signals do not function on corticosteroids such as hydrocortisone [46]. Pathogen related yeast (PRY) proteins are capable of secreting hydrophobic sterols across the cell membrane and are endogenous to *S. cerevisiae* [8]. Pry 1 and Pry 2 are secretory glycoproteins and members of the cysteine-rich secretory proteins, antigen 5, and pathogenesis related 1 proteins (CAP) superfamily [8]. These 2 PRY proteins have the conserved CAP domain in their C-terminal [8]. See Appendix A for the coding sequences. Sterol binding is a conserved function of the CAP superfamily, allowing for sterol secretion. Given the hydrophobic nature of hydrocortisone, the sterol-secreting ability of PRY proteins may allow for its secretion along with other corticosteroids. Increasing the expression of Pry 1 and Pry 2 will theoretically increase the rate of hydrocortisone secretion. This can be achieved by including the genes within the plasmid to express the proteins in addition to their native production.

The translational elongation factor EF-1 alpha (TEF1) promoter sequence from *S. cerevisiae* is used to maximize hydrocortisone production and avoid metabolic overload [46]. TEF1 is naturally used to express the protein Tef1p in abundance in *S. Cerevisiae* [46]. Given the need for abundant Tef1p expression, TEF1 is designed to be a strong promoter which can be utilized for hydrocortisone. The NAD-dependent glycerol-3-phosphate dehydrogenase (GPD1) promoter is another strong promoter, like TEF1 [46]. However, GPD1 is noticeably stronger than TEF1, which may result in excessive hydrocortisone production and a non-optimal pathway balance in the *S. cerevisiae* cells [47]. Hydrocortisone produced using the TEF1 promoter has no toxic effects on the *S. cerevisiae* cells, indicating no need for any additional regulatory regions such as induced promoters [48]. The TEF1 promoter region better supports sustainable hydrocortisone production by optimizing the product without exhausting the *S. cerevisiae* cells' metabolic resources. See Appendix A for the full TEF1 promoter sequence.

The coding sequence will be optimized by replacing rare codons in the inserted genes. *S. cerevisiae* has a bias for certain base pairs compared to mammals [49]. This increases the number of rare codons which require rare tRNAs, decreasing the efficiency of translation. Given the use of human genes to code for enzymes such as CYP17A1 needed for hydrocortisone production, rare bases should be replaced with analogous common bases. For example, the most common human codon for valine is GUG which is the rarest valine codon in *S. cerevisiae* [50]. Therefore, GUG should be replaced by GUU, the most common in *S. cerevisiae*, for increased

efficiency in any human genes expressed by the yeast [50]. This should be done for all codons which are not commonly present in *S. cerevisiae*.

The Tsynth27 [9] terminator region is used to optimize hydrocortisone production in *S. cerevisiae* yeast cells. The CYC1 terminator is most used in *S. cerevisiae* genetic engineering, however shorter synthetic terminators have shown higher performance [51]. Tsynth27 is 60 bp in length, compared to the CYC1 terminator with a length of 261 bp [52]. Short synthetic terminators were used to express a heterologous enzyme in *S. cerevisiae* that enables the metabolic production of itaconic acid [51]. The Tsynth27 terminator region showed the highest production of itaconic acid when compared to the native CYC1 terminator [51]. The differences in itaconic acid produced were slight, however synthetic terminators may further enhance the metabolic pathway performance when paired with a strong promoter [51]. Additionally, using a synthetic terminator allows for more precise control of gene expression, leading to improvements in metabolic engineering [51]. See Appendix A for the genetic sequence of the Tsynth27 terminator.

4.0 Cell Cultivation Methods

4.1 Growth Medium Composition

4.1.1 Nutritional Requirements

The *S. cerevisiae* strain used to produce hydrocortisone requires a carbon source, nitrogen, essential vitamins, minerals, and trace elements for optimal growth and survival. Glucose is the most efficient carbon source, as it is preferred by yeast due to its high glycolytic flux and strong catabolite repression [5]. Glucose is more readily available and a cost-effective source of carbon for the yeast strain. Additionally, *S. cerevisiae* requires a supply of nitrogen, which is essential for amino acid and nucleotide synthesis. Ammonium sulfate is the common source of nitrogen because it is directly incorporated into the biosynthetic pathways of the *S. cerevisiae* cell without additional breakdown. It has a high nitrate content and maintains the pH of the medium, *S. cerevisiae* specifically requires a pH of 6.5 [5]. Additionally, the sulfate component contributes to the buffering capacity in the medium. Essential vitamins are regularly used to help the growth of our cell [4]. Biotin, pantothenic acid, and inositol in the yeast extract are added to improve the efficiency of the growth and yield. Finally, hydrocortisone production using *S. cerevisiae* cells involves metabolic engineering to enable steroid biosynthesis, often using P450 monooxygenases [5]. This requires oxygen, iron, zinc, and copper as cofactors [4]. Additionally,

efficient lipid metabolism depends on magnesium, calcium, and manganese content in the medium [4].

4.1.2 Medium Composition

The base medium composition for *S. cerevisiae* engineered for hydrocortisone biosynthesis is listed below in Table 1.

Table 1: Growth Medium Composition for Recombinant *S. Cerevisiae*

Component	**Quantity (per liter)**	**Justification**
Glucose	20 g	The primary source of carbon [17].
Yeast Nitrogen Base (YNB)	6.7 g	Provides essential vitamins and trace minerals [17].
Ammonium sulfate	5 g	A source of nitrogen [17].
Citric acid	4.8 g	Part of the citrate-phosphate buffer maintaining the pH [20], [21].
Dibasic disodium phosphate	7.1 g	Part of the citrate-phosphate buffer maintaining the pH [20], [22].
Magnesium sulfate	0.5 g	A cofactor for ATP –dependent enzymes [17].
Calcium chloride	0.1 g	Used for membrane stability [17].
Sodium chloride	0.1 g	Used to maintain osmotic balance [17].
Vitamin mixture	0.5 mg	Contains vitamins B1, B2, B3, B5, B6, B7 and inositol, all vital.
Folic acid	0.2 µg	Used for nucleotide synthesis [23].
Trace element solution	1 mL	Contains iron, zinc, copper, manganese, sodium and boric acid [23].

The listed components of the base medium ensure that the *S. cerevisiae* has sufficient nutritional content and support cofactors to allow for optimal growth and yield efficiency. Glucose is added to the mixture because *S. cerevisiae* is a glucophillic yeast, meaning it prefers glucose over fructose as a carbon source [53]. Maltose is not a viable sugar option as the maltose metabolism in *S. cerevisiae* is regulated by glucose repression, meaning the consumption of maltose is much lower compared to glucose [54]. Finally, the retail price range of glucose is between $3.86 USD and $5.79 USD per kilogram [55], and fructose is between $2.94 USD and $7.83 USD per kilogram [55], making glucose more economically viable.

4.2 Reactor Type

A continuous stirred-reactor tank (Chemostat CSTR) is best suited to hydrocortisone production through fermentation of *S. cerevisiae* yeast cells [2]. *S. cerevisiae* requires precise control over reactor parameters to optimize hydrocortisone production, making a well-controllable bioreactor essential. Turbidostat reactors operate with a fluctuating environment and respond to cell density changes, whereas Chemostat reactors maintain a contestant steady-state environment, optimal for *S. cerevisiae* [56]. Stirred-tank bioreactors (STRs) are a practical choice for this process due to their efficient mixing, oxygen transfer, and their ability to accurately regulate environmental conditions. Effective mixing is essential to maintaining optimal yeast metabolism by ensuring the uniform distribution of nutrients and oxygen [5]. The CSTR provides uninterrupted stirring inside the reactor, allowing for increased control of the mixture's oxygenation and accessibility to the required nutrients. The CSTR regulates other environmental parameters essential for yeast growth and metabolism as well, such as temperature and pH . Other reactors, such as the plug flow reactor (PFR), have difficulties managing the temperature within the reactor due to their lack of mixing [10]. Since the *S. cerevisiae* cells require a precise temperature window between 25°C to 30°C, the CSTR is the ideal reactor for maintaining this specific range and optimizing yeast growth and metabolism. Additionally, the CSTR is significantly easier to maintain and clean compared to the PFR, which is challenging due to its intricate design and narrow channels [57]. Using a reactor that is easy to maintain, allows precise control of parameters, and ensures the *S. cerevisiae* cells receive necessary nutrients and oxygen will enhance the efficiency and performance of the process. Therefore, a CSTR is the clear and preferred choice.

4.2.1 Reactor Operating Mode

The most suitable reactor operating mode to produce hydrocortisone is continuous. A continuous process is preferred for steady, high-volume, large-scale applications, such as the production of hydrocortisone [3]. A batch process is not suitable since there must be a continuous feed of sugar to the yeast in the reactor. To add, there is no method to modify nutrient levels in response to cell activity in a batch reactor [3]. A continuous operating mode was chosen over a fed-batch mode to accommodate the large-scale production of hydrocortisone, as a continuous process is better suited for sustaining high output. Additionally, continuous processes can achieve higher yields by reducing manufacturing interruptions, such as the frequent cleaning and sterilization required

between batch runs [39]. Studies conducted on the fermentation of *S. cerevisiae* showed that a continuous process yielded 18% higher concentration of products (108.14 g/L vs. 91.36 g/L) and more than triple the productivity (14.71 g/L/h vs. 4.57 g/L/h) than the batch process [58]. To drive the production of hydrocortisone, the growth media is added continuously to the CSTR.

4.2.2 Design Considerations

A Rushton radial flow impeller is used to stir the CSTR. Flat, vertically set blades are set along an agitation shaft to produce a unidirectional radial flow [59] This is preferred over the axial flow impeller because the lateral movement facilitates more thorough mixing, and S. *cerevisiae* can tolerate the increased shear stress [60]. The reactor has a layer of insulating foam, to maintain the ideal operating temperature and prevent heat loss [61]. A citrate phosphate buffer, $(C_6H_5O_{11}P)^{-6}$, is included in the growth medium to control the pH levels, this buffer has been chosen over others such as potassium sulphate that can prevent enzyme activity [6]. Additionally, a pH probe continuously monitors the reactor's pH levels. If the pH deviates from the desired range, a control loop is in place to add extra buffer as needed. A control loop manages the volume in the reactor. A sensor signals if the volume in the reactor is abnormal, then a flowrate control valve placed on the growth media inlet stream can close if the volume is too high, or open if it is too low. To maintain the desired dissolved oxygen levels, a dissolved oxygen sensor controls an inflow of sterile air that is fed into the tank through a sparger at the bottom. Sterile air is an inexpensive alternative to pure oxygen, as it only needs to be filtered to remove contaminants and heated to kill possible pathogens, this will be done at the processing facility. Additionally, the other components in the air do not affect our process [62].

4.2.3 Reactor Schematic

Shown below is a drawn-out schematic of how all the components described above come together. It is a conceptual drawing that doesn’t consider any sizing or proportions.

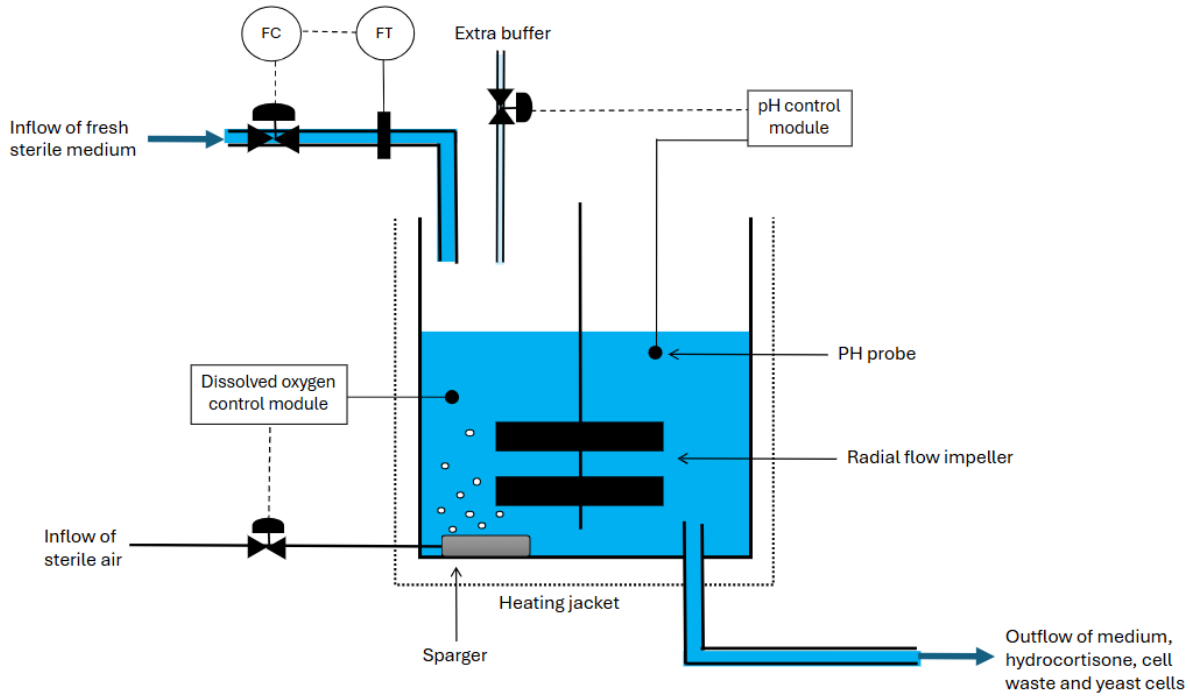

Figure 4: Reactor Schematic Highlighting Key Features and Control Systems [63]

4.3 Operating Parameters

4.3.1 Operating Conditions

The reactor is kept at 25°C with a pH of 6.5 and limited exposure to light. In a study of two strains of *S. cerevisiae*, a maximum growth rate was achieved at 30°C, with high growth rates between 25°C to 30°C [38]. In optimal conditions, this achieves a cell doubling time of 1.5 to 2 hours [4]. In glycerol producing yeast, the maximum production rates were found in the same temperature range [38]. Hydrocortisone is unstable as temperature increases. Over 35 days, solutions of hydrocortisone and a citrate buffer lost 16.52 ± 2.64% of their original concentration at 20°C to 22°C, while there was only an 8.77 ± 1.26% decrease at 3°C to 7°C [64]. Therefore, 25°C was selected as the reaction temperature to maximize the growth and production rate of the yeast while attempting to minimize hydrocortisone degradation. 25°C is within the optimal growth and production range while not drastically exceeding the degradation seen at 20°C to 22°C. Degradation rates were at a minimum when a citrate buffer of pH 6.5 was used in comparison to lower pHs or sterilized water [64]. Maximum glycerol production rates in yeast were seen between a pH of 5.92 and 6.27 [38]. Therefore, a pH of 6.5 is used to maximize product stability while being close to optimal production conditions. Light exposure inhibits

yeast cell growth and transport in cells expressing genes for cytochromes b, a, and a_3 [65]. Light is also seen to degrade hydrocortisone [64]. Therefore, light exposure should be reduced as much as possible to promote growth and product recovery.

Yeast can be grown at high density, with cell densities reaching up to 100g/L of dry weight [4]. A maximum hydrocortisone production of 677 mg/L was observed at an optical density 600 of 180 [66]. This is incredibly dense and may not be feasible on an industrial scale. Given higher density is correlated with higher hydrocortisone production, a cell density of 100g/L is targeted. The concentration of the media within the reactor is kept constant to allow for the yeast to focus on production rather than adaptation. Therefore, the media has the same concentrations described in **Section 4.1**.

4.3.2 Control Method

Production and cell growth reaches a maximum at 30°C while product stability decreases past 22°C [38], [64]. Therefore, cooling is necessary for temperatures more than 30°C. In case of overheating due to the exothermic nature of the metabolic reaction, a cooling fluid would be used at 2°C. Heat transfer is more efficient when there is a temperature difference of at least 10°C to drive heat flow, allowing for suitable heat transfer to occur. Cooling the reactor to 20°C does not harm cells as it only decreases the production rate, ensuring that production continues once it is returned to operating temperature. Additionally, 20°C is roughly the ambient temperature which decreases utility costs.

The pH should not increase above 7 or drop below 6 to protect the hydrocortisone. Yeast can withstand a wide variety of pH conditions, but the hydrocortisone degrades more rapidly if not kept close to 6.5 [64]. The components of the citrate-phosphate buffer, sodium phosphate dibasic dihydrate and citric acid, can be used to balance the pH [6]. Sodium citrate is basic with a pH around 9 (5% solution) and can be added when the pH decreases past 6 to return to the optimal range [67]. Citric acid has a pKa of 2.63 at 25°C and can be used to lower the pH if it exceeds 6.5 pH [68]. Using the components of the buffer to adjust the pH ensures the physical properties of the buffers remain constant. Solutions should be diluted to reduce changes in overall concentration to prevent high osmotic pressure.

Given the nature of a continuous process, media must always be added into the system to maintain steady-state conditions. The concentration of vitamins, minerals, and glucose in the feed can be adjusted to maintain a constant concentration within the reactor. In the chemostat reactor, maintaining steady-state conditions is typically achieved through feed composition monitoring and control [69]. To do so, online sensors would be used to monitor media conditions such as oxygen levels and glucose concentration in real-time [70]. These sensors enable automated control loops that can adjust feed rates or composition, allowing the constant control of the reactor contents [71]. This real-time monitoring is essential to the core control scheme of a chemostat, where stable growth conditions is critical [69].

4.3.3 Sterility

The process is at risk of contamination. The substrates used to grow yeast can be used by many other organisms. Given the yeast doubling time is 1.5 to 2 hours, bacteria and other microbes can propagate much faster and overtake yeast cultures [4], [72]. The greatest risk of this contamination is through the continuously added media. Media should be sterilized through indirect heating using two heat exchangers on the media feed line [72]. Sterilization occurs at temperatures above 100°C [72]. The time spent at 100°C must be experimentally determined to effectively sterilize the media, while not causing degradation [72]. The aerobic nature of the recombinant *S. cerevisiae* means that air must be fed into the reactor which also requires sterilization. Deep-bed filtration is the most readily used method, allowing for effective removal of micro-organisms through highly porous fibers between 5 to 30 microns in diameter [30]. While effectively filtering air, there is a large pressure drop across the filter which could lead to high pressures within the reactor [72]. A bleed valve should be added onto the reactor in order to release excess air. The positive pressure flowing out from the bleed valve prevents any contamination from unfiltered air.

5.0 Product Separation Methods

5.1 Target Product Specifications

Hydrocortisone is kept as a water-soluble powder and diluted by medical practitioners directly before use [16]. Therefore, the product will be produced as a powder directly to reduce the

amount of processing required after purification. The product must be highly purified given its various uses in humans such as direct injections. The product will be purified to at least 98% hydrocortisone as this is the industry standard in pharmaceuticals [17],[18],[19]. The US Pharmacopeia requires hydrocortisone to make up 97 to 100% of the dry basis of a sample to be considered for medical use [20]. Therefore, 98% purity is sufficient for US regulations and meets the industry standard.

5.2 Process Flow Diagram and Process Decisions

The continuous process to produce hydrocortisone with a purity of >98% is outlined below and corresponds to the Process Flow Diagram (Figure 5) in section 5.3.

5.2.1 Centrifugal Separation

Stream 1, containing the mixed slurry from the bioreactor, enters the AKW hydrocyclone for the first separation step. Like a centrifuge, the hydrocyclone uses centrifugal force to separate liquids of different densities. However, the hydrocyclone can accomplish this with a simpler and cheaper device. Additionally, the AKW hydrocyclone was shown to have higher separation efficiency in separating yeast strains from fermentation broth when compared to other industry-grade hydrocyclone equipment [21]. The *S. cerevisiae* yeast strain leaves the hydrocyclone as waste in Stream 2 and the fermentation broth in Stream 3 continues to the secondary separation step.

5.2.2 Thermal Precipitation

The secondary separation step involves the precipitation of hydrocortisone by thermal precipitation [73]. This process will occur in a continuous decanter which facilitates solid-liquid separation within one single continuous process [74]. By cooling Stream 4 to 2°C in a continuous decanter, crude hydrocortisone will precipitate and float to the bottom of the stream [29], [30]. The primarily liquid upper section of the stream will exit the decanter as waste in Stream 6. Meanwhile, the lower portion of the stream containing hydrocortisone and a reduced amount of fermentation broth will continue in Stream 5.

5.2.3 High Performance Liquid Chromatography (HPLC)

Stream 7, containing growth medium and crude hydrocortisone, enters a high-performance liquid chromatography (HPLC) for further purification. HPLC is used to further separate the hydrocortisone product from remaining solvents. Liquid chromatography separates molecules in a liquid mixture by passing them through a column with a stationary phase. The mixture's components interact differently with the stationary and mobile phases, leading to their separation [22]. The HPLC unit requires a few other components including a pump to provide solvent flow and a built-in column oven to ensure the temperature remains stable for optimized separation. A solvent degasser is used in the HPLC to remove any remaining air and CO_2 through Stream 8 [22]. A detector is a beneficial add-on to this unit, since it measures the separated analytes as they exit the column and generates a signal output for any monitoring software. The HPLC unit will operate around 30°C since most HPLC columns operate at ambient temperature [23].

Reversed-phase HPLC columns are commonly used to purify corticosteroids (hydrocortisone included) through separation [24]. The separation medium in reverse-phase HPLC is non-polar and interacts with the polar mobile phase solvent. A modified non-polar silica, specifically ODS-silica (Octadecylsilane-modified silica), is used as the stationary phase medium. ODS-silica efficiently and accurately separates hydrocortisone from other solvents in high performance liquid chromatography [24]. Silica has a high mechanical strength, allowing it to withstand pressures in HPLC without collapsing [25] and it is thermally stable [26]. Additionally, silica particles provide high surface area and controlled pore volume, allowing for better interactions with the analytes in the solvent to improve overall separation [27]. The separated hydrocortisone exits the HPLC in Stream 9 along with waste in Stream 8 containing media and ethanol.

5.2.4 Crystallization

After HPLC, hydrocortisone from Stream 9 is dissolved in propylene glycol from Stream 10, acting as a primary solvent. This occurs at a temperature of 50°C to increase the solubility of hydrocortisone [28]. Once dissolved, distilled water is added through Stream 13 and the temperature is reduced to 2°C in a cooler unit to lower solubility and supersaturate the hydrocortisone [29], [30]. The solution is then fed into a semicontinuous constant stir tank where it is agitated to promote the crystallization of hydrocortisone from the supersaturated solution [29], [31]. The remaining solution is removed through Stream 15, and the crystals are rinsed with

distilled water to remove particles adsorbed to the hydrocortisone through Stream 17. The solution is fed into a fines trap and recycled back into the cooler while the crystallized hydrocortisone continues through Stream 18 [31]. This crystallization process is repeated a second time (Streams 19 to 27) to improve hydrocortisone purity to greater than 98%. The purified hydrocortisone exits through Stream 27.

5.2.5 Drying

The purified hydrocortisone in Stream 27 enters the desiccator which is attached to a vacuum pump. The solution of hydrocortisone and water is then vacuum-dried at a temperature of 40°C to remove all moisture in Stream 28 [75]. The vacuum is a full vacuum and held at 25mmHg to reduce the evaporation temperature of any remaining propylene glycol and water [31] After exiting the vacuum dryer, the product is cooled to 25°C. The result is the finished hydrocortisone in a desirable white, powder state in Stream 30 with a purity of >98%.

5.3 Process Flow Diagram (PFD)

The Process Flow Diagram presented below contains the steps to the continuous process for producing hydrocortisone with >98% purity. Leaving the bioreactor, the fermentation broth enters an AKW hydrocyclone for primary separation and a liquid chromatograph for secondary separation. Next, crude hydrocortisone is crystallized and then recrystallized for greater purity. Finally, the hydrocortisone is dried and produced in the desired powder form.

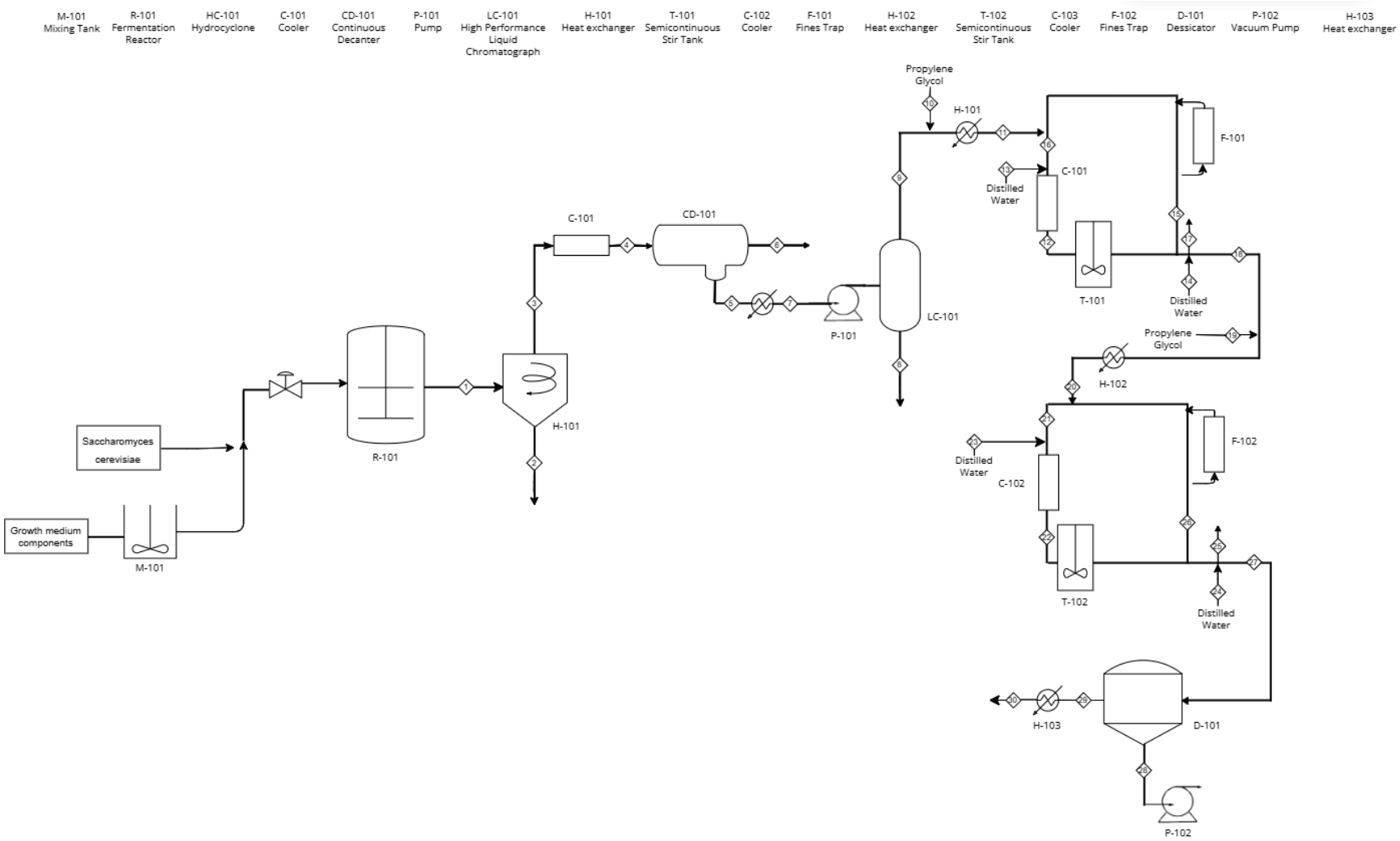

Figure 5: Process Flow Diagram of Hydrocortisone Production Process

Table 2: Stream table describing the components of each stream of the Process Flow Diagram (Figure 5)

Stream Number	1	2	3	4	5	6	7	8	9	10
Temperature (°C)	25	25	25	2	2	2	30	25	25	25
Pressure (mmHg)	760	760	760	760	760	760	760	760	760	760
Components										
Hydrocortisone	Present	0	Present	Present	Present	0	Present	0	Present	0
S. Cerevisae	Present	Present	0	0		0		0	0	0
Water	Present	Present	Present	Present	Present	Present	Present	Present	0	0
Growth Medium	Present	Present	Present	Present	Present	Present	Present	Present	Present	0
Cell Waste	Present	Present	Present	Present	Present	0	Present	Present	0	0
CO2	Present	Present	Present	Present	Present	Present	Present	Present	0	0
Air	Present	Present	Present	Present	Present	Present	Present	Present	0	0
Ethanol	Present	Present	Present	Present	Present	Present	Present	Present	0	0
Propylene Glycol	0	0	0	0	0	0	0	0	0	Present

Stream Number	11	12	13	14	15	16	17	18	19	20
Temperature (°C)	50	2	2	25	2	30	25	2	25	50
Pressure (mmHg)	760	760	760	760	760			760	760	760
Components										
Hydrocortisone	Present	Present	0	0	Present	Present		Present	0	Present
S. Cerevisae	0	0	0	0	0	0		0	0	0
Water	0	Present	Present	Present	Present	Present	Present	Present	0	Present
Growth Medium	Present	Present	0	0	0	Present	Present	0	0	0
Cell Waste	0	0	0	0	0	0		0	0	0
CO2	0	0	0	0	0	0		0	0	0
Air	0	0	0	0	0	0		0	0	0
Ethanol	0	0	0	0	0	0		0	0	0
Propylene Glycol	Present	Present	0	0	Present	Present	Present	Present	Present	Present

Stream Number	21	22	23	24	25	26	27	28	29	30
Temperature (°C)	30	2	2	25	25	25	25	40	40	25
Pressure (mmHg)	760	760	760	760	760	760	760	25	25	25
Components										
Hydrocortisone	Present	Present	0	0	0	Present	Present	Present	Present	Present
S. Cerevisae	0	0	0	0	0	0	0	0	0	0
Water	Present	Present	Present	Present	Present	Present	Present	Present	0	0
Growth Medium	0	0	0	0	0	0	0	0	0	0
Cell Waste	0	0	0	0	0	0	0	0	0	0
CO2	0	0	0	0	0	0	0	0	0	0
Air	0	0	0	0	0	0	0	0	0	0
Ethanol	0	0	0	0	0	0	0	0	0	0
Propylene Glycol	Present	Present	0	0	Present	Present	Present	Present	0	0

5.0 Conclusion

The production of hydrocortisone using genetically modified *S. cerevisiae* yeast strains presents an efficient and scalable solution to meet the growing demand for this steroid. By applying the metabolic engineering strategies presented, including gene insertion, codon optimization and enhanced secretion mechanisms, the yeast strain can be modified for more efficient hydrocortisone biosynthesis and higher product yield. The production of hydrocortisone from engineered *S. cerevisiae* requires specific growth medium composition, reactor design and operating parameters to optimize yield. By implementing a chemostat continuous stirred-tank reactor and maintaining the desired parameters, this method offers an efficient and scalable process to hydrocortisone biosynthesis. The hydrocortisone produced using this process meets US Pharmacopeia regulations and US industry standards. After departing the reactor, the slurry commences a series of centrifugal separation, thermal precipitation and liquid chromatography to obtain crude hydrocortisone. Following repeated crystallization for increased purity and vacuum drying, hydrocortisone in a desirable powder form with a purity of greater than 98% is produced.

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Appendix A

Promoter Sequence

TEF1 / YPR080W [43]**:** (1377 bp)

ATGGGTAAAGAGAAGTCTCACATTAACGTTGTCGTTATCGGTCATGTCGATTCTGGTAAGTCTACCACT
ACCGGTCATTTGATTTACAAGTGTGGTGGTATTGACAAGAGAACCATCGAAAAGTTCGAAAAGGAAGC
CGCTGAATTAGGTAAGGGTTCTTTCAAGTACGCTTGGGTTTTGGACAAGTTAAAGGCTGAAAGAGAAA
GAGGTATCACTATCGATATTGCTTTGTGGAAGTTCGAAACTCCAAAGTACCAAGTTACCGTTATTGATG
CTCCAGGTCACAGAGATTTCATCAAGAACATGATTACTGGTACTTCTCAAGCTGACTGTGCTATCTTGA
TTATTGCTGGTGGTGTCGGTGAATTCGAAGCCGGTATCTCTAAGGATGGTCAAACCAGAGAACACGCT
TTGTTGGCTTTCACCTTGGGTGTTAGACAATTGATTGTTGCTGTCAACAAGATGGACTCCGTCAAATGG
GACGAATCCAGATTCCAAGAAATTGTCAAGGAAACCTCCAACTTTATCAAGAAGGTTGGTTACAACCC
AAAGACTGTTCCATTCGTCCCAATCTCTGGTTGGAACGGTGACAACATGATTGAAGCTACCACCAACG
CTCCATGGTACAAGGGTTGGGAAAAGGAAACCAAGGCCGGTGTCGTCAAGGGTAAGACTTTGTTGGA
AGCCATTGACGCCATTGAACAACCATCTAGACCAACTGACAAGCCATTGAGATTGCCATTGCAAGATG
TTTACAAGATTGGTGGTATTGGTACTGTGCCAGTCGGTAGAGTTGAAACCGGTGTCATCAAGCCAGGT
ATGGTTGTTACTTTTGCCCCAGCTGGTGTTACCACTGAAGTCAAGTCCGTTGAAATGCATCACGAACAA
TTGGAACAAGGTGTTCCAGGTGACAACGTTGGTTTCAACGTCAAGAACGTTTCCGTTAAGGAAATCAG
AAGAGGTAACGTCTGTGGTGACGCTAAGAACGATCCACCAAAGGGTTGCGCTTCTTTCAACGCTACCG
TCATTGTTTTGAACCATCCAGGTCAAATCTCTGCTGGTTACTCTCCAGTTTTGGATTGTCACACTGCTCA
CATTGCTTGTAGATTCGACGAATTGTTGGAAAAGAACGACAGAAGATCTGGTAAGAAGTTGGAAGAC
CATCCAAAGTTCTTGAAGTCCGGTGACGCTGCTTTGGTCAAGTTCGTTCCATCTAAGCCAATGTGTGTT
GAAGCTTTCAGTGAATACCCACCATTAGGTAGATTCGCTGTCAGAGACATGAGACAAACTGTCGCTGT
CGGTGTTATCAAGTCTGTTGACAAGACTGAAAAGGCCGCTAAGGTTACCAAGGCTGCTCAAAAGGCTG
CTAAGAAATAA

Expression Cassette Sequence

PRY1 / YJL079C [46]: (900 bp)

ATGAAACTTTCTAAATTATCGATCTTAACCTCCGCCTTAGCCACGTCTGCATTGGCCGCTCCTGCCGTT
GTTACTGTCACTGAACACGCCCATGAGGCTGCAGTCGTTACTGTGCAAGGCGTAGTTTATGTCGAAAA
CGGCCAAACACGTACAACTTACGAAACACTAGCTCCTGCTTCCACTGCCACTCCAACTTCTACAGCTAC
AGCTTTGGTTGCTCCCCCTGTCGCTCCTTCCTCTGCTTCCAGCAATTCCGATGTGGTCTTGTCTGCTTTG
AAGAACTTAGCCTCTGTTTGGGGTAAAACTACCGATTCAACGACCACGTTGACATCTTCTGAATCTACA
TCGCAATCGTTGGCTCAAGCTACTACGACTTCTACACCCGCTGCTGCTTCCACCACTTCCACACCCGCT
GCTACCACCACCACTTCTCAAGCCGCTGCTACTAGCTCAGCATCGTCTTCGGATAGTGACCTGTCAGAT
TTTGCCTCTTCTGTGTTGGCTGAACACAACAAGAAGAGAGCTTTGCACAAGGACACACCAGCTTTGTC
CTGGTCCGATACTTTGGCCTCCTACGCTCAAGACTATGCTGACAACTATGATTGCTCCGGCACTTTGAC
CCATTCTGGCGGTCCATACGGTGAAAACTTGGCTTTGGGTTATGACGGCCCAGCTGCCGTCGACGCTTG
GTACAATGAAATTTCCAACTACGACTTCTCGAATCCAGGCTTTTCAAGTAACACTGGCCACTTTACTCA
AGTCGTTTGGAAGTCCACCACCCAAGTTGGTTGTGGCATCAAAACCTGTGGCGGTGCATGGGGTGACT
ATGTCATCTGTAGTTACGACCCCGCAGGAAACTACGAAGGCGAATACGCCGATAATGTCGAGCCCCTA
GCTTAA

PRY2 / YKR013W (Genomic) [46]: (990 bp)

ATGAAATTTTCTAAAGTCTCACTACTGGCCGCATCTGCATCTGTCGCTTTATCTGCCCCAGTTGCTGTG
ACTGTCACGCAACATGTCCACCAAGCTGCCACTGTAGTGGTACAAGGTATAGTTCGTGTAGAAAATGG
CCAGACTCTAACGACGTTCATTACCAAGGGCACTCAAACGGCTTCTGCTAGTCCCGTTGCCACAACAT
CAGCTCCTATTGTGGTTGCTAATGCTCAAGTGGACAGCATAGCTACTTCCGTTATCCAAGAAAGCGCTG
TCGTGGCTGAATCTGCAACATTTGAAGAATCCTCTACAGAAACATCTGAAGCATTTTCTACAGCAACT
GCAACCATACAAGCGGTGCAAACCTCCGCAAGTGCCACACAGGATGATGTAACCACAACTTTAACATC
TTCAACACAACCTACCAGCACAACTACTCCAACAACCACTACCACTAGCCCAACCACTACCACTAGCC
CAACTACTACCGCTAGTCCAACTACTACTGCTAGCCCAACCACCGCTACCACCACTCAATCTACCGCCT
CAAGCACTCAATCCTCAAGCTCCGATTTCTCAACGTCAATGGTTAACGAACACAACACTAAAAGGGCG
TTGCACAAGGATACCGGTTCTTTGACATGGTCTGACACACTAGCAACATATGCACAAAACTACGCTGA
CTCCTACGATTGTTCAGGCAACCTAGTCCACTCCGGCGGTCCATACGGTGAAAACTTGGCACTTGGTTA
CGGTACGACCGGCTCTGTTGATGCCTGGTATAACGAAATTACCAGCTACGACTATTCCAACCCTGGTTT
TAGTGAAAGCGCAGGTCACTTCACCCAAGTCGTATGGAAGGGAACTTCTGAAGTTGGTTGCGGTTTGA
AATCTTGCGGTGGCGAATGGGGCGATTACATCATTTGTTCCTACAAAGCCGCTGGTAATGTCATTGGTG
AGTTTGCTGACAACGTTATGCCATTAGCTTAA

NADPH-Cytochrome P450 Reductase / pgGYR (Genomic Sequence) [76]: (2097 bp)**NADPH-Cytochrome P450 Reductase / pgGYR (Genomic Sequence)** [77]: (2097 bp)

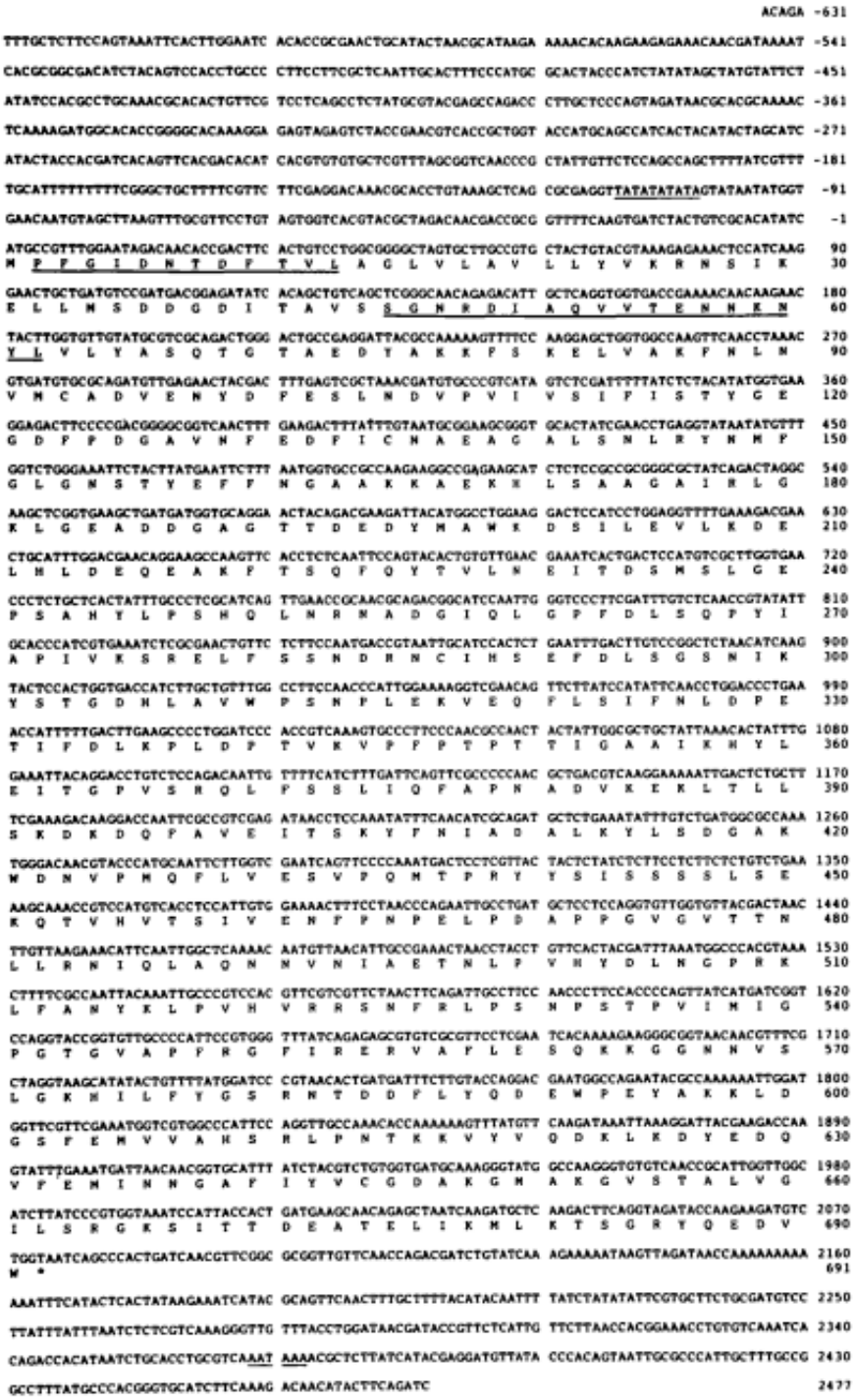

Figure A1: Genomic sequence of NADPH-Cytochrome P450 Reductase from Yabusaki et al.

Terminator Sequence

Tsynth27 [9]**:** (60 bp)

TGGGTGGTATATATATATATATATATATATATAACTGTCTAGAAATAAAGAGTATCATCTTTCAAA