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Research Idea

Using N_2 as a final electron receptor in yeast to produce NH_3 in bioreactors

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Abstract

This research proposal aims for the replacement of N_2 molecule (instead of O_2) as a final electron receptor in micro-organisms so that they can produce NH_3, which is important industrially, instead of H_2O. This idea is based on the hypothesis that replacement of the central atom in gas-carrying compounds in living organisms such as chlorophyll, heme and hemocyanin would replace the fixed gas. However, NH_3 is an expensive product because it is produced using the Haber-Bosch reaction technique which requires high temperature and pressure. Therefore, it is predictable here to produce NH_3 so simply in bioreactors which is a great economic benefit.

Keywords

nitrogen fixation; chlorophyll; heme; ammonia, ferrochelatase and adaptive mutation.

Objectives

NH_3 (ammonia or azane gas) is an important product; it has a number of vital uses such as fertilisers, cleaners, fermentation industry and antimicrobial agent in food products. NH_3 is currently produced using the Haber-Bosch reaction technique which requires high temperature (400-500°C) and pressure (150-250 bar) which is why it is so expensive, so

the production of ammonia gas in our proposed way would provide a great economic benefit.

Impact

1. Production of NH_3 in bioreactors (low-cost), instead of using the Haber-Bosch reaction technique (high-cost).
2. Scientific achievement: If our purpose succeeded, our new yeast strain, would be the first of its type; we would have an organism that "breathes" N_2, instead of O_2 for the first time.
3. Our new yeast strain would start new scientific horizons in terms of evolution, microbiology, adaptive mutations and life itself.

Introduction/Background

Photosynthesis and respiration are two vital processes that are the prerequisites for all life on Earth. The two pigments responsible for these processes are chlorophyll and heme, respectively. The so-similarity between chlorophyll and heme has been a mysterious subject of research by some researchers for a long (Vernon and Seely 1966); they are identical in structure except for the central atom: magnesium in chlorophyll and iron in heme (Bryson and Vogel 1965). Chlorophyll has been proven to have the ability to turn into haemoglobin (Hughes and Lanter 1936, Kohler et al. 1939).

O_2 is fixed by living organisms to be used as a final electron receptor, to be turned into H_2O. If we managed to replace O_2 by N_2 in yeast (because yeast contains heme) and green bacteria, we are going to obtain NH_3 and that is a real economic benefit. NH_3 (ammonia or azane gas) is an important product; it has a number of vital uses such as fertilisers, cleaners, fermentation industry and antimicrobial agent in food products. NH_3 is currently produced using the Haber-Bosch reaction technique which requires high temperature (400-500°C) and pressure (150-250 bar) which is why it is so expensive (Mike 2024), so the production of ammonia gas in our proposed way would be a great economic benefit.

Heme is the compound that forms, with some proteins, haemoglobin, the compound that exists in red blood cells in most animals. It has the ability to deliver O_2 and CO_2. Its chemical structure is $C_{55}H_{72}N_4Fe$. Hemocyanin is the compound that delivers O_2 and CO_2 in mollusc and arthropod species the same way hemin does, but less effectively. Its chemical structure is $C_{55}H_{72}N_4Cu$. Chlorophyll is the compound that fixes O_2 and CO_2 in plants. It also absorbs sun light's energy necessary for photosynthesis. Its chemical structure is $C_{55}H_{72}N_4Mg$. Hemin has a semi-similarity with other compounds such as cobalamin (vitamin B_{12}) which has the Co atom as a central atom in its structure.

Research Approach and Methodology

Accurately, gases do not bind heme; as a whole, they bind only the central atom (iron) within it. It makes a sense that, if we managed to change the central atom in heme, the fixed gas would be changed. When we have breath in our lungs, only oxygen binds haemoglobin, not because oxygen is more suitable than other gases, but because of the central atom in heme, iron, which has a high affinity with oxygen.

Iron exists naturally in the form of oxides i.e. FeO, Fe_2O_3, Fe_3O_4. Copper, in hemocyanin, exists in nature in the form of oxides too i.e. Cu_2O CuO, CuO_2, Cu_2O_3. Magnesium, in chlorophyll, also interacts with oxygen and carbon dioxide easily. Therefore, our hypothesis is "Changing the central atom in haemoglobin, hemocyanin and chlorophyll, changes the fixed gas by them".

While some researchers managed to replace the central atom in chlorophyll with copper and zinc *in vitro* (Petrovic et al. 2006), no attempts have been made to replace the central atom in chlorophyll and/or heme in living organisms for two reasons, according to the author's point of view. They are:

1. The absence of purpose, which is available in this proposal; bio-production of ammonia gas.
2. Technical complexity, which may seem to be impossible at first sight for two reasons. Firstly, hemin and chlorophyll are synthesised through a set of bioreactions, so if you need to do a specific modification in heme, for instance, you will need to make a number of "specific" genetic alterations or mutations in yeast, for instance, which sounds to be impossible. Secondly, if you replaced the central atom in heme with Ca^{2+} *in vitro*, obtained Ca-Heme and managed to replace it with the heme of yeast (using microinjection technique), your new trait would not be inherited by next generations because of the ferrochelatase enzyme which is selective to Fe ions (Ogun et al. 2023).

Ferrochelatase (or protoporphyrin ferrochelatase) is an enzyme which is encoded by the FECH gene in humans (Anonymous In Press). It catalyses the terminal (8^{th}) step in the biosynthesis of heme, turning protoporphyrin IX into heme B. The interesting fact (to this proposal) is that ferrochelatase can also insert other divalent metal ions into protoporphyrin. Some ions such as Zn^{2+}, Ni^{2+} and Co^{2+} form other metalloporphyrins (Medlock 2009). Zinc protoporphyrin (ZPP) is a compound found in red blood cells when heme production is inhibited by lead and/or by lack of iron (Labbe et al. 1999). Substrate inhibition happens in the order $Cu^{2+} > Zn^{2+} > Co^{2+} > Ni^{2+}$ and can be alleviated by supplementation of metal complexing agents, such as β-mercaptoethanol or imidazole to the reaction buffer (Hunter et al. 2008). Magnesium-chelatase is a three-component enzyme that catalyses the insertion of Mg^{2+} into protoporphyrin IX. This is the first unique step in the synthesis of chlorophyll and bacteriochlorophyll (Willows 2003, Bollivar 2006).

Although it is possible to replace the central atom in heme and chlorophyll, it looks very difficult to make specific genetic manipulations to reach the target of our proposal.

Therefore, our goal can be possible if we follow an adaptive mutation technique. Adaptive mutation states that, rather than mutations and evolution being random, they are responsive to certain stresses. In other words, the mutations which happen are more beneficial and specific to the given stress, neither random nor a response to anything in particular. The term stress refers to any alteration in the environment, such as temperature, nutrients, population size etc. Tests with microorganisms have found that, for adaptive mutation, more of the mutations noted after a given stress were found to be more efficient at dealing with the stress than chance alone would suggest is possible (Foster 1993, Sniegowski and Lenski 1995). Adaptive mutation is a very controversial topic so there have been many experiments to prove or discredit the theory. Three key examinations are the SOS response (McKenzie 2000), responses to starvation in Escherichia coli (Foster 2000) and testing for revertants of a tryptophan auxotroph of *Saccharomyces cerevisiae* (Foster 2000).

Proposed experiment

Model of research:

- The micro-organism(s) that we are going to use in our experiment must have two properties:

1. Obligate aerobic: If a facultative aerobic micro-organism was used, it is highly probable to simply turn into anaerobic pathway when aerobic conditions are not suitable to it.
2. Contains heme or bacteriochlorophyll.

- The advantages of using a yeast strain over using a bacterium strain as a model of research are:

1. Ferrochelatase adds ferrous ion as a terminal step in heme biosynthesis, while magnesium chelatase adds magnesium ion as a first step in bacteriochlorophyll synthesis; therefore, if any modification was made in magnesium chelatase enzyme, it may be required to be followed by further modifications in the enzymes that synthesise bacteriochlorophyll which is more difficult. It is much easier to modify the top of a pyramid than modifying its base.
2. The biosynthesis of heme looks simpler than the biosynthesis of bacteriochlorophyll. Besides, the structure of ferrochelatase is simple and its molecular weight is low relative to magnesium chelatase.

On the other hand, mutation rate in prokaryotes, in general, is much higher than mutation rate in eukaryotes which is a disadvantage of using a yeast strain over using a bacterium strain as a model of study in this proposal. Therefore, it is proposed here to use a yeast strain and a bacterium strain at the same time to avoid the need to use any mutants that may induce unpredictable, undesired mutations. *Torulopsis psychrophile*, *T. austromarina*, *L. gelidum* and *L. nivalis* are examples for obligate aerobic yeasts (Anonymous 2023), so they are candidates to be models for this study.

Fe^{2+}, Cu^{2+} and Co^{2+} are naturally selected by ferrochelatase, all lying in the fourth group of the periodic table. They are all bivalent and their electronegativity ranges from 1.83 to 1.90. Besides, they are all reactive to O_2. Therefore, if we are to replace ferrous ions at the hemin of yeast, we should focus on an element that is:

1. Bivalent.
2. Have in-range or close electronegativity.
3. Reactive to N_2.

As previously mentioned, Zn^{2+} is naturally selected by ferrochelatase (causing a disease in humans). Its electronegativity is 1.65 and it is reactive to N_2 forming Zn_3N_2. Thus, Zn^{2+} looks to be perfect to be our first candidate to be replaced by ferrous ions in hemin. The electronegativity of Ni^{2+} is 1.91. It is reactive to N_2 (forming Ni_3N), so it would be a good fit to be our second candidate. At the third place, comes Mn^{2+}. Its electronegativity equals 1.55. It is also reactive to N_2 forming Mn_3N_5, Mn_3N_2 and MnN_2.

Proposed experiment steps:

- In a lab fermenter, *Torulopsis psychrophile*, for instance, should be grown on a suitable medium with the replacement of iron with zinc under normal aerobic conditions. It is predictable here that Zn-heme will be synthesised in the yeast cells.
- pH value of the medium may be needed to be modified several times because ferrochelatase selectivity is pH-dependent (Hunter et al. 2008).
- N_2 gas should be replaced by the same volume of O_2 gas gradually as a trial to induce an adaptive mutation.
- N_2 is less reactive than O_2 (bond energy of oxygen equals 119 k.cal/mole, but equals 226 k.cal/mole for nitrogen), so temperature may be needed to be increased. As a result, the yeast strain to be chosen as a model of research should be thermophilic or superthermophilic.
- Test the aerobic environment of the bioreactor for ammonia gas periodically. If found, we have reached our goal.
- Mutants may be needed to be used.

Ethics and security

It is recommended to apply Biosafety Level 2 (BSL-2) (Anonymous 2015) during working on this experiment and to keep our (to be) new yeast strain "safe" in the laboratory. The new yeast strain must not be used for commercial purposes before carefully studying the risks and/or the advantages of its possible spread in nature. If there would be high risks associated with its possible spread, its employment for commercial purposes would not be advised.

Conflicts of interest

The authors have declared that no competing interests exist.

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