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H_2 PRODUCTION AND ROLE OF HYDROGENASES IN *ESCHERICHIA COLI* BATCH CULTURES DURING FERMENTATION OF MIXTURE OF GLYCEROL AND ACETATE AT DIFFERENT pHs

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E. coli is able to ferment sugars and/or glycerol for producing H_2. H_2 is produced via four multiple and reversible [Ni-Fe] Hyd enzymes. This study describes growth and H_2 production in batch cultures during utilization of mixture of acetate (5 g/l) and glycerol (10 g/l) at various pHs (7.5, 6.5) in *E. coli* wild type and mutants with defects in Hyd genes.

It has been determined that in batch tests at pH 7.5 and 6.5 wild type strain evolved H_2 during ~168 h. Interestingly, it was shown *hyaB* and *hybC* single mutants have exhibited the same results at bot pHs; especially H_2 generation was ~ 150 h. This is prolonged period compared to acetate alone fermentation.

Taken together, it can be concluded that cell growth and H_2 generation depends on external pH and carbon sources. Particularly, Hyd-1 and Hyd-2 work towards H_2 oxidation which is in contrast to glycerol only fermentation suggesting that acetate affects Hyd enzymes working direction. The data suggest that in these conditions Hyd-3 with Hyd-4 are major Hyd enzymes responsible for H_2 at pH 6.5. But it is opposite at pH 7.5, where Hyd-3 only is major. It is assumed that low pH is inhibitory for growth of bacterial cells. The data are significance for biofuel, especially for biohydrogen production technology, when using mixture of different carbon sources.

Escherichia coli – acetate and glycerol fermentation – bacterial growth – hydrogenases – pH

E. coli-ն կարող է խմորել շաքարներ և / կամ գլիցերոլ H_2 արտադրելու համար: H_2 ար-տադրվում է չորս դարձելի [Ni-Fe] Հիդ ֆերմենտների միջոցով: Այս ուսումնասիրությունը նկա-րագրում է աճը և H_2 արտադրությունը քացախաթթվի (5 գ/լ) և գլիցերոլի (10 գ/լ) խառնուրդի օգտագործման ընթացքում աղիքային ցուպիկի վայրի տիպի և տարբեր Հիդ-ների գեներում առկա խախտումներով մուտանտներում տարբեր pH-ներում (7.5, 6.5):

Ցույց է տրվել, որ pH 7.5 և 6.5-ում վայրի տիպում H_2 առաջացել է մինչև ~168 ժ տևողու-թյամբ: Հետաքրքիր է, որ *hyaB* և *hybC* մուտանտներն ունեցել են նույն արդյունքները pH 7.5 և 6.5-ում, և, հատկապես, H_2 արտադրվել է մինչև ~150 ժ: Սա երկար ժամանակ է՝ համեմատած միայն քացախաթթվի խմորման հետ:

Կարելի է եզրակացնել, որ բջիջների աճը և H_2 արտադրությունը կախված են արտաքին pH-ից և ածխածնի աղբյուրից: Մասնավորապես, Հիդ-1-ը և Հիդ-2-ն աշխատում են H_2 օքսիդացման ուղղությամբ, ի տարբերություն միայն գլիցերոլի խմորման, որտեղից ենթադրվում է, որ քացախաթթուն ազդում է Հիդ ֆերմենտների աշխատանքի վրա:

Ստացված տվյալները ցույց են տվել, որ այս պայմաններում և pH 6,5-ում H_2-ի համար պատասխանատու են Հիդ-3 և Հիդ-4 ֆերմենտները: Սակայն pH 7.5-ում, կարևոր է միայն Հիդ-3-ը: Պարզվեց, որ ցածր pH-ն ունի արգելակիչ ազդեցություն բակտերիաների բջիջների աճի վրա:

Ստացված արդյունքները կարևոր նշանակություն ունեն կենսավառելիքի, հատկապես կենսաջրածնի արտադրության տեխնոլոգիայի համար, երբ օգտագործվում են ածխածնի աղբյուրների տարբեր խառնուրդներ:

Escherichia coli – քացախաթթվի և գլիցերոլի խմորում – բակտերիաների աճ – հիդրոգենազներ – pH

E. coli способна утилизировать сахара и/или глицерин для получения H_2.H_2 с помощью четырех обратимых ферментов [Ni-Fe] Гид. Это исследование описывает рост и производство H_2 в периодических культурах во время использования смеси ацетата (5 г/л) и глицерина (10 г/л) при различных pH (7,5; 6,5) у кишечной палочки дикого типа и у мутантов с дефектами в генах Гид.

Было определено, что при pH 7,5 и 6,5 у дикого штамма H_2 производилась в течение длительного времени до ~168 ч. Интересно, что у *hyaB* и *hybC* мутантов наблюдаются те же результаты при pH 7,5 и 6,5; особенно генерация H_2 до ~150 ч. Это длительный период по сравнению с отдельной ферментацией ацетата.

Было показано, что рост клеток и генерация H_2 зависят от внешнего pH и источника углерода. В частности, Гид-1 и Гид-2 работают в направлении окисления H_2 в отличие от ферментации глицерина. Предполагается, что ацетат влияет на работу Гид-ов. Полученные результаты свидетельствуют о том, что в этих условиях Гид-3 с Гид-4 являются основными ферментами, ответственными за производство H_2 при pH 6,5, однако при pH 7,5 только Гид-3 ответствен за производство H_2.

Предполагается, что низкий pH является ингибитором для роста бактериальных клеток. Эти данные имеют большое значение для производства биотоплива, особенно для технологии производства биоводорода при использовании смеси различных источников углерода.

Escherichia coli – ферментация ацетата и глицерина – рост бактерий – гидрогеназы – pH

Renewable and sustainable biofuel production is an important goal: in particular molecular hydrogen, which is alternative, renewable and ecologically clean energy source [10]. *E. coli* is one of the best studied microorganisms for hydrogen production because genetic manipulation is developed, as well as, the biochemistry of many metabolic pathways for enhanced hydrogen production is understood [13]. *E. coli* produces hydrogen by dark fermentation under anaerobic conditions when no external electron acceptors are present [9]. This process is catalyzed by special membrane-associated enzymes named hydrogenases; *E. coli* has four membrane bound reversible [Ni-Fe] Hyd enzymes. Hyd-1 and Hyd-2 encoded by the *hya* and *hyb* operons, respectively, are mainly H_2 uptake enzymes [8]. Hyd-3 and Hyd-4 encoded by the *hyc* and *hyf* operons, respectively, are mainly H_2 producing enzymes and have similarities with each other [6]. In addition, it has been confirmed that the activity of Hyd enzymes depends on external pH [11], that is why were used different pHs. This is very important property for understanding the regulatory mechanism of enzyme activity and thus enhancing H_2 production.

It is well known that *E. coli* can ferment different carbon sources like sugars (glucose, lactose) acetate and glycerol, and the mixtures of which are available in many agricultural and industrial wastes [2]. As it is well known glycerol is main waste of biodiesel industry that is why it is very cheap source for producing H_2 compared to sugars. In 2006 it has been discovered that *E. coli* is able to produce H_2 from glycerol, when the fermentation is conducted at pH 6.3 [3].

Nowadays for enhancing H_2 production experiments are on-going to use different mixtures of carbon sources like sugars and glycerol as cheap carbon is present in the wastes. In the study is presented the relationship of four Hyd enzymes both with each other and in the cycle of producing H_2. Particularly, the role of acetate and glycerol on ORP kinetics and H_2 production was investigated in single *hyaB, hybC, hycE, hyfG* (lacking large subunits of Hyd-1, Hyd-2, Hyd-3, Hyd-4, respectively), double *hycE hyfB-R* (lacking large subunits of Hyd-3 and subunits of Hyd-4) and triple *hyaB hybC hycE* (lacking large subunits of Hyds 1-3) mutants during growth in bacterial culture at both alkaline and acidic pHs up to ~200 h.

Materials and methods.

1. *Bacterial strains and growth conditions*

E. coli BW25113 wild type and mutant strains with deletions in the genes coding subunits for different Hyd enzymes were used in the study. The strains used are listed in tab. 1.

Table 1. Characteristics of *E. coli* wild type and mutant strains used

Strains	Genotype	Absent hydrogenase subunit or related protein	References
BW25113	*$lacI^q$ $rrnB_{T14}$ $\Delta lacZ_{W116}$ hsdR514 $\Delta araBAD_{AH33}$ Δrha BAD_{LD78}*	wild type	[15]
MC4100	*F-araD139 D (argF-lac)U169 l-rpsL150 relA1deoC1 flhD5301 D(fruK-yeiR)725(fruA25) rbsR22 D(fimBfimE) 632 (::IS1)*	wild type	[1]
JW0955 Km^{Ra}	BW 25113 *ΔhyaB*	large subunit of Hyd-1	[14]
JW2962 Km^{Ra}	BW 25113 *ΔhybC*	large subunit of Hyd-2	[14]
JW 2691Km^{Ra}	BW 25113 *ΔhycE*	large subunit of Hyd-3	[15]
JW2472 Km^{Ra}	BW25113 *ΔhyfG*	large subunit of Hyd-4	[14]
JRG 3633	BW25113 *ΔhycE ΔhyfB-R;*	large subunit of Hyd-3, subunits B-R of Hyd-4	[7]
BW25113 ΔhyaB ΔhybC ΔhycE	BW25113 *ΔhyaB ΔhybC ΔhycE*	large subunits of Hyd-1, Hyd-2 and Hyd-3	[4]
FTD147	MC4100 *ΔhyaB ΔhybC ΔhycE*	large subunits of Hyd-1, Hyd-2 and Hyd-3	[12]

[a]resistant to kanamycin

Bacteria were grown anaerobically in batch for 18-22 h culture at 37^0C. Bacteria from an overnight growth culture were added (1.5 %) into the fresh peptone medium containing 20 g/l peptone, 15 g/1 K_2HPO_4, 1.08 g/l KH_2PO_4, 5 g/l NaCl (pH 7.5), 20 g/l peptone, 7.4 g/1 K_2HPO_4, 8.6 g/l KH_2PO_4, 5 g/l NaCl (pH 6.5) and 20 g/l peptone, 1.08 g/1 K_2HPO_4, 15 g/l KH_2PO_4, 5 g/l NaCl (pH 5.5) and added with acetate (2 g/l), and glycerol (10 g/l) as carbon sources. During bacterial growth DCCD (0.2 mM) was added, which is an inhibitor of the membrane-associated enzyme F_0F_1-ATPase.

The medium pH was measured by a pH-meter (HI-3220, Hanna Instruments, Romania) using a glass body combination double-junction pH electrode and adjusted by 0.1 M NaOH or 0.1 N HCl.

The bacterial biomass growth was studied with the help of spectrophotometer (UV-VIS sprectrophotometer, Cary 60, Agilent Technologies, USA) monitoring the OD readings of bacterial culture absorbance under 600 nm. The bacterial SGR (μ) stated, as lg2/doubling time, was calculated where the logarithm of OD was growing linearly with time.

2. *The ORP and H_2 determination of during bacterial growth*

ORP of bacterial suspension and H_2 production were measured using the glass body refillable ORP electrode Pt BNC (HI-3131, Hanna Instruments, Portugal). This electrode is sensitive to H_2 or O_2, and its readings drop to negative values (> - 400 mV) confirmed the H_2 formation in the medium under anaerobic conditions. ORP, pH and OD measurements were monitored during ~200 h.

During the growth of *E. coli* H_2 production was confirmed by the appearance of gas bubbles in the test tubes over the bacterial medium with the help Durham tubes, and it was verified by chemical reaction of $KMnO_4$ solution in H_2SO_4 with H_2, as before [14].

3. ***Reagents and data processing***

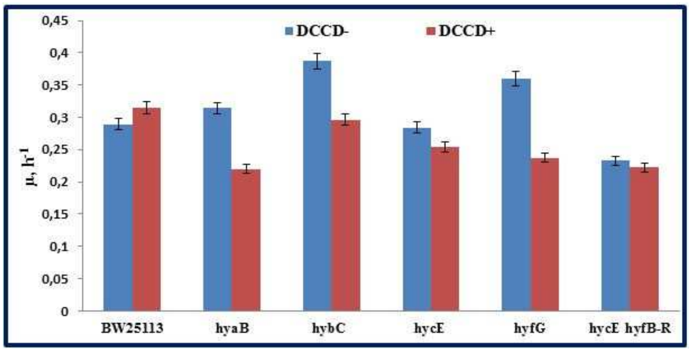
Acetate, glycerol, agar, peptone (Carl Roths GmbH, Germany) and other reagents of analytical grade were used in the study. Three independent experiments were done and the average data were calculated with the standard errors, and mention was only made for over 3 % The validity of differences between experimental and control data was evaluated by Student's criteria (p) [5]; $p < 0.01$ or less if this is not represented, otherwise $p > 0.5$ if the difference was not valid.

Results and Discussion.

1. Growth and H_2 production of E. coli wild type and mutants with defects in Hyd-1 to 4 during mixed carbon fermentation in assays supplemented with acetate and glycerol at pH 7.5

In *E. coli hyaB* and *hycE* strains was the same SGR compared with wild type at pH 7.5 (fig. 1A). In *hycE hyfB-R* the SGR of bacterial cells was decreased ~1.2 fold, however in *hybC* and *hyfG* mutant strains the SGR was increased ~1.3 and ~1.2 fold, respectively, compared with wild type (fig. 1 A). It was determined the SGR in the presence of DCCD inhibitor, during which only in wild type was stimulated a little, but in all mutant strains the DCCD had an inhibitory effect. DCCD had high inhibitory effect in *hyaB*, *hybC* and *hyfG* single mutant strains ~ 1.4, ~1.3, ~1.5 fold, respectively (fig. 1 A). This can suggest that F_0F_1-ATPase has a role in bacterial growth at pH 7.5.

A



B

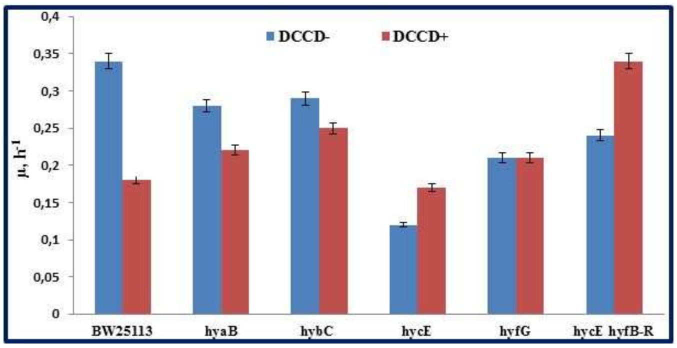
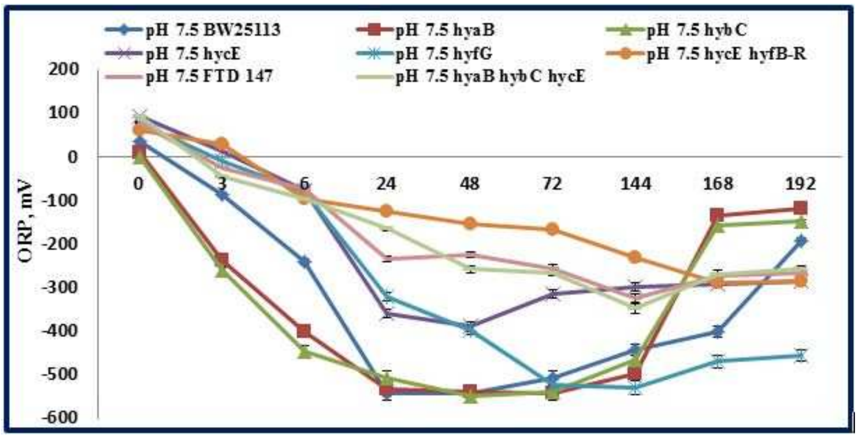


Fig. 1. Specific growth rate (μ) of *E. coli* BW25113 wild type and mutants with defects in Hyd-1 to Hyd-4 during mixed carbon fermentation in the assays supplemented with acetate and glycerol at pH 7.5 (A) and pH 6.5 (B). For mutant strains see tab.1.

It was determined that H_2 production was produced in *hyaB* and *hybC* single mutant strains from 6 h, however in wild type from 24 h (fig. 2 A). In *hyfG* mutant strain the H_2 production started from 48 h and lasted up to 200 h. It suggests that *hyfG* is the best mutant strain in this condition for H_2 production. However it was not detected in *hycE*, double and triple mutant strains suggesting that Hyd-3, but not Hyd-4, is responsible for H_2 production during fermentation of acetate and glycerol at pH 7.5 (fig. 2 A).



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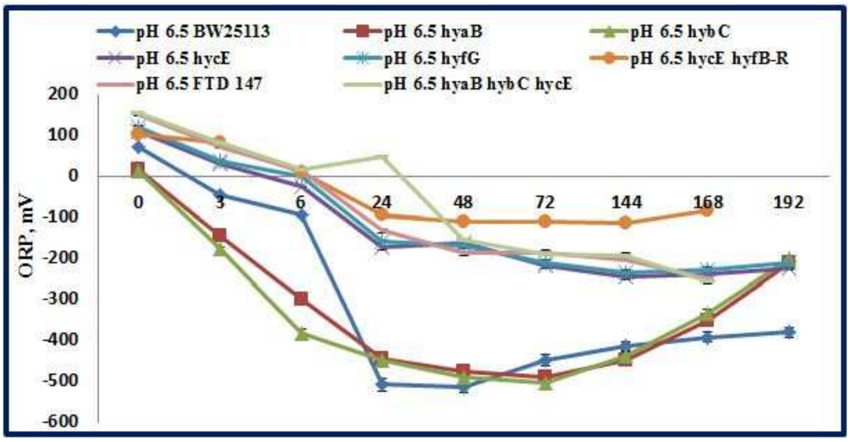
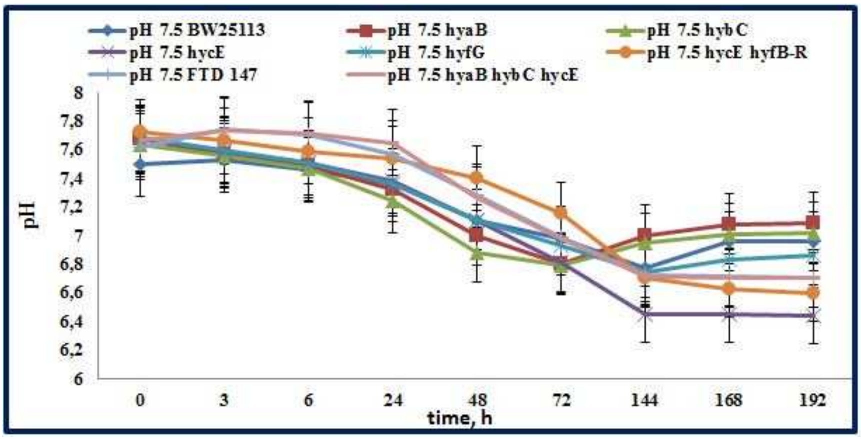


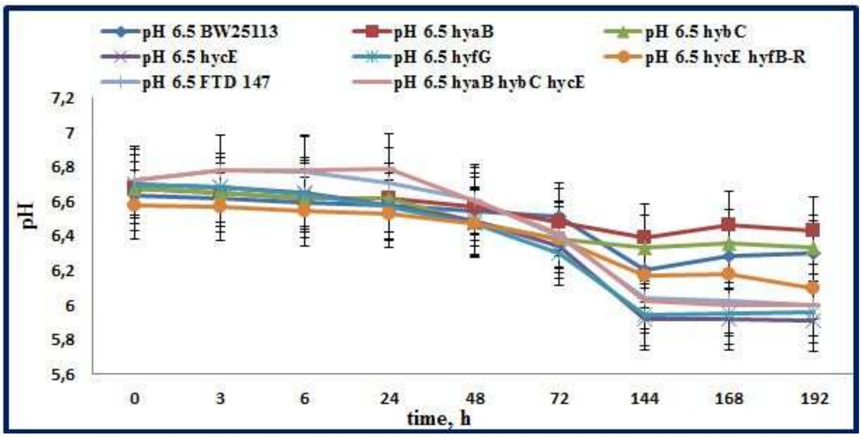
B

Fig. 2. The kinetics of ORP and H_2 production of *E. coli* BW25113 wild type and mutants with defects in Hyd-1 to Hyd-4 during mixed carbon fermentation in the assays supplemented with acetate and glycerol at pH 7.5 (A) and pH 6.5 (B). For mutant strains see tab. 1.

It was determined the external pH changes during growth at pH 7.5. In wild type, *hycE*, *hyfG*, double and triple mutant strains the pH was decreased by ~0.5 unit up to 144 h, after that it was constant (fig. 3 A). However, in *hyaB* and *hybC* mutant strains pH was decreased by ~0.7 unit up to 72 h and then it was increased up to 200h (fig. 3 A).



A



B

Fig. 3. The external pH changes of *E. coli* BW25113 wild type and mutants with defects in Hyd-1 to Hyd-4 During mixed carbon fermentation in the assays supplemented with acetate and glycerol at pH 7.5 (A) and pH 6.5 (B). For mutant strains see tab.1.

2. *Growth and H_2 production of E. coli wild type and mutants with defects in Hyd-1 to 4 during mixed carbon fermentation in assays supplemented with acetate and glycerol at pH 6.5*

The maximum SGR was determined in wild type, but in *hyaB*, *hybC*, *hyfG*, *hycE hyfB-R* mutants the SGR was decreased ~1.2, ~1.2, 1.6 and ~1.4 fold, respectively (fig. 1B). The SGR in *hycE* mutant strain was decreased ~2.8 fold, which suggests, that

Hyd-3 has an important role during acetate and glycerol fermentation at pH 6.5. DCCD had inhibitory effect on SGR in wild type, *hyaB* and *hybC* single mutants ~1.9, ~1.2 and ~1.2 fold, respectively. But SGR was stimulated ~1.4 fold in *hycE* and *hycE hyfB-R* mutants compared to wild type (fig. 1 B).

The H_2 production was determined in wild type from 24 h, but in *hyaB* and *hybC* mutant strains it was detected from 6 h, which suggests that the absence of Hyd-1 and Hyd-2 enzymes had negative effect on H_2 production generation time (fig. 2 B). It was not shown for *hycE* and *hyfG* mutant strains, which suggests that Hyd-3 and Hyd-4 are responsible for H_2 production at pH 6.5. The double and triple mutants clarified that Hyd-3 and Hyd-4 together are responsible for H_2 production (fig. 2 B).

pH monitoring was determined during growth of bacterial cells also at pH 6.5 (fig. 3 B). In all assays pH dropped by ~0.4 unit (fig. 3 B). Therefore acids were generated in the mediums.

At pH 5.5 no H_2 production was determined in all assays during acetate and glycerol utilization. It suggests that pH is important for H_2 production and pH 5.5 is not optimal condition during fermentation of mixture of acetate and glycerol.

In this study *E. coli* Hyd activity and H_2 production were studied at various pHs during mixed carbon (acetate and glycerol) fermentation. Different *E. coli* Hyd mutants were used during mixed carbon fermentation for discovering the role of Hyd enzymes in *E. coli* at various pHs. The results suggest that all Hyd enzymes can either work in H_2 uptake or producing directions depending on added carbon source and external pH. In this case mainly Hyd-3 is responsible for H_2 production at pH 6.5, but opposite effect at 7.5: Hyd-3 with Hyd-4 are major Hyd enzymes responsible for H_2. H_2 was produced earlier and SGR was higher mainly in *hybC* mutant strain. No any effect was observed in all strains at pH 5.5 (the data are not present). It seems that growth of bacterial cells was inhibited at low pHs.

In *hyaB* or *hybC* mutants H_2 production was detected earlier than in wild type at pH 7.5. But at pH 6.5 only in *hybC* mutant earlier H_2 production was detected suggesting important role of Hyd-2 under these conditions. In *hyfG* mutant strain the H_2 production started from 48 h, but was not detected in *hycE*, double and triple mutant strains at pH 7.5. These results indicate that Hyd-3, but no Hyd-4, is responsible for H_2 production during fermentation of acetate and glycerol at pH 7.5. Moreover, the data suggest that for H_2 production Hyd-3 and Hyd-4 are together responsible at pH 6.5.

The data are significant in biofuel production technology, especially for H_2 production using bacteria when applying mixture of carbon sources.

Abbreviations

ATPase- Adenosinetriphosphatase
DCCD- *N,N′-dicyclohexylcarbodiimide*
E. coli- Escherichia coli
H_2- molecular hydrogen
Hyd- hydrogenase
OD- optical density
ORP- oxidation-reduction potential
SGR- specific growth rate

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