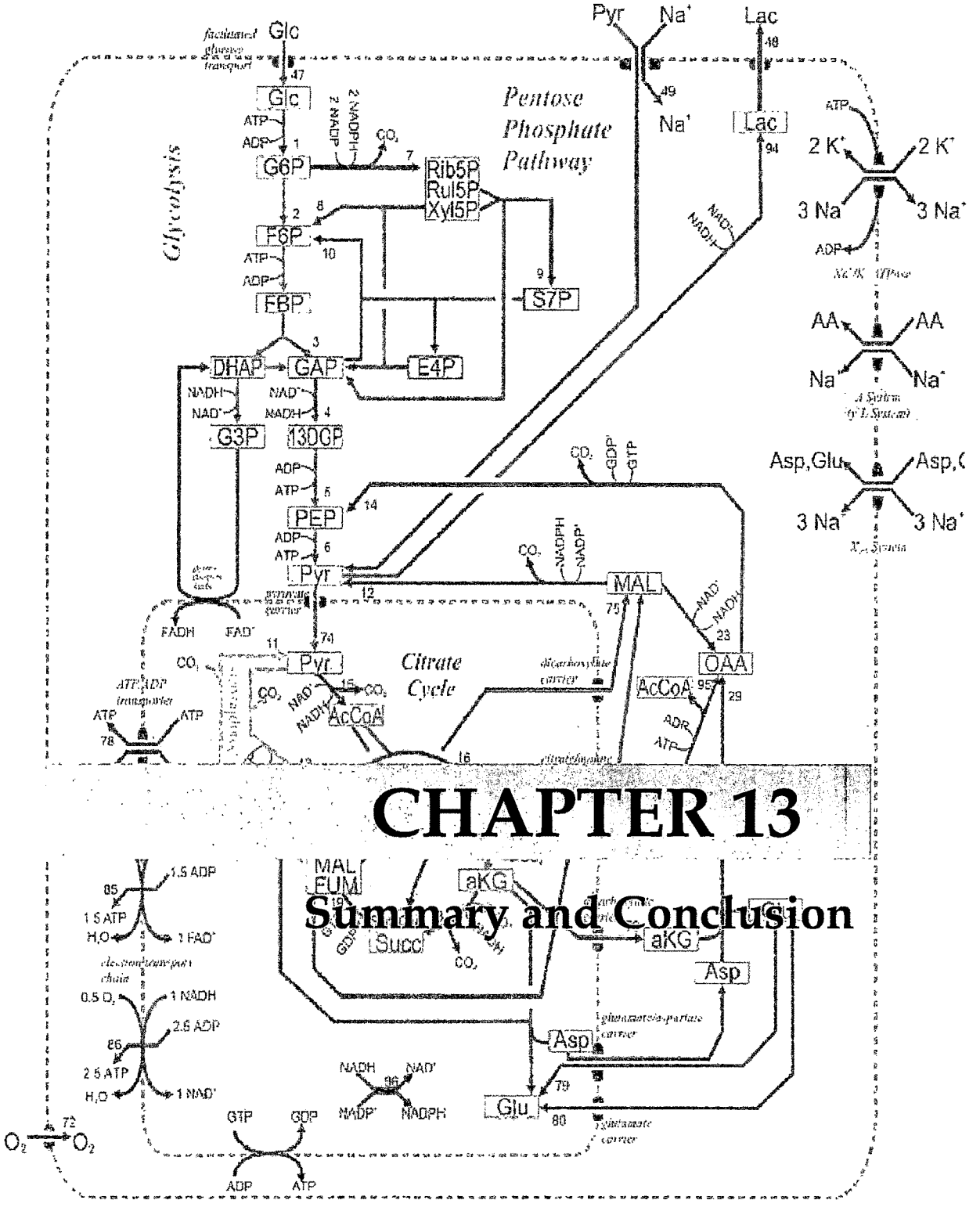

CHAPTER 13

Summary and Conclusion

Post genomic era has benefitted metabolic engineering by generating vast information on various metabolic pathways operating in the cell and also helped in designing tools for successfully modulating these pathways to obtain the desired product. *E. coli* has been one of the most preferred organisms for metabolic engineering. The present study investigates the effect of genetic modifications at the OAA-Pyruvate-PEP node and TCA in *E. coli* K and *E. coli* B strains for citrate production. Strategies employed were conditional down regulation of *icd* gene using antisense regulated by P_{fruB} and P_{tac} promoters coupled with *ppc* and *gltA* gene overexpression. *E. coli* MA1935 an *icd* mutant was used as a model to validate the strategy employed.

Chapters 3-6 demonstrate the construction of as-*icd* under P_{fruB} promoter and its effect on the metabolism of *E. coli* DH5α and *E. coli* BL21. Down regulation of ICDH activity by ~ 3-4 fold through P_{fruB} as-*icd* expression in *E. coli* DH5α had no deleterious effect on growth but demonstrated an increased glucose consumption rate and CS activity. Increased CS activity accompanied with citrate accumulation. Chapter 3 and 4 demonstrated that use of 100 mM glucose (excess) resulted in acetate outflow which is detrimental for high levels protein synthesis in *E. coli* DH5α; hence lesser glucose concentration was used.

Parameters	*E. coli* DH5α	*E. coli* BL21
Growth rate	0.26 ± 0.02	0.54 ± 0.04
Glucose consumption rate	7 ± 1.5	6.7 ± 0.72
Glucose consumed	48 ± 2.5	45.5 ± 4.0
G-6-PDH	0.13 ± 0.08 U	0.18 ± 0.03 U
CS	0.035 ± 0.002 U	0.05 ± 0.006 U
ICDH	0.30 ± 0.02 U	1.01 ± 0.09 U
ICL	ND	0.007 ± 0.002
Citrate (Intracellular)	0.65 ± 0.06 mM	1.206 ±0.04 mM
Acetate (100 mM glucose)	34 ± 1.5 mM	27.67 ± 0.42 mM
Acetate (50 mM glucose)	ND	20 ± 3 mM

Table 13.1: Physiological and metabolic differences between *E. coli* DH5α and *E. coli* BL21 on glucose.

Alternatively *E. coli* BL21 strain was used for these genetic manipulations as it is known to secrete lower amounts of acetate on excess glucose due to altered Cra regulation leading to constitutive functioning of glyoxylate and gluconeogenic pathways. Physiological and metabolic differences between *E. coli* DH5α and *E. coli* BL21 on glucose are given in **Table 13.1.** *E. coli* DH5α demonstrated slower growth rate compared to *E. coli* BL21. Moreover enzyme activities of CS, ICDH, G-6-PDH and ICL were higher in *E. coli* BL21 on glucose while high ICDH activity on acetate in *E. coli* BL21 supported alteration in Cra regulation. Moreover the citrate accumulation was also high in *E. coli* BL21 compared to *E. coli* DH5α as a result of high TCA flux in *E. coli* BL21. Chapter 6 demonstrates P_{fruB} as-*icd* is ineffective in *E. coli* BL21(λDE3) as P_{fruB} was not regulated on glucose which further supports the presence of altered regulation of Cra (**Table 12.2**).

Parameters	*E. coli* DH5α pVS2k3	*E. coli* BL21(λDE3) pVS2k3	*E. coli* BL21 pJE6
Growth rate	No change	No change	No change
Glucose consumption rate	~2 fold increase	-	~ 1.5 fold decrease
Glucose consumed	No change	-	~ 1.2 fold decrease
G-6-PDH	~ 1.2 fold decrease	No change	No change
CS	~ 2 fold increase	No change	~ 1.2 fold increase
ICDH	~3-4 fold decrease	No change	~ 3-4 fold decrease
ICL	ND	No change	No change
Citrate (Intracellular)	~ 2 fold increase	No change	~ 2 fold increase
Acetate	~ 2 fold increase	No change	~ 1.5 fold decrease

Table 13.2: Effect of decrease in ICDH activity using P_{fruB} as-*icd* (pVS2k3) and P_{tac} as-*icd* (pJE6) in *E. coli* DH5α and *E. coli* BL21.

~ 3-4 fold decrease in ICDH activity in *E. coli* BL21 was achieved by using as-*icd* under P_{tac} promoter (Chapter 7, **Table 13.2**) which lowered glucose consumption rate and glucose consumed but no alteration in growth. These results were contrary to the as-*icd* expression in *E. coli* DH5α. Decrease in ICDH activity also increased CS activity

with concomitant ~ 2 fold increase in citrate accumulation and decreased acetate secretion by ~ 1.5 fold.

E. coli strains lack citrate transport system; hence in the present study citrate transporter (H^+ citrate transporter) from *Klebsiella pneumoniae* was expressed under P_{lac} promoter in combination with ICDH down regulation to facilitate citrate efflux and to overcome the deleterious effect of citrate accumulation. Overexpression of citrate transporter in *E. coli* DH5α expressing P_{fruB} as-*icd* had decreased ICDH activity by ~ 2-3 fold and demonstrated poor growth rate and glucose consumption rate. It also had increased CS and G-6-PDH activity coupled with increased citrate accumulation and acetate secretion.

Parameters	*E. coli* DH5α pVS2k3:pVSD1	*E. coli* BL21 pJE6:pVSD1
Growth rate	~ 1.5 fold decrease	~ 1.5 fold increase
Glucose consumption rate	~ 5 fold decrease	No change
Glucose consumed	No change	No change
G-6-PDH	~ 1.5 fold increase	No change
CS	~ 1.2 fold increase	~ 2 fold increase
ICDH	~ 2-3 fold decrease	~ 2-3 fold decrease
ICL	ND	~ 1.2 fold decrease
Citrate (Intracellular)	~ 2 fold increase	~ 2 fold decrease
Acetate	~ 0.5 fold increase	No change

Table 13.3: Effect of decrease in ICDH activity using P_{fruB} as-icd (pVS2k3) and P_{tac} as-*icd* (pJE6) in *E. coli* DH5α and *E. coli* BL21 in presence of citrate transporter.

In contrast to *E. coli* DH5α, *E. coli* BL21 expressing P_{tac} as-*icd*, in presence of citrate transporter increased growth rate but without any alteration in glucose consumption rate, glucose consumed and G-6-PDH activity. Decrease in ICDH activity also increased CS activity and decreased ICL activity. Increase citrate accumulation was observed without any change in acetate secretion (**Table 13.3**). Hence, Chapter 5 and 7 described that down regulation of *icd* gene increased CS activity resulted in citrate

accumulation in both the strains although significant differences were found with respect to growth rate, glucose consumption rate and various enzyme activities. Incorporation of citrate transporter did not show any extracellular citrate secretion in both the *E. coli* strains.

Parameters	*E. coli* BL21(λDE3)	*E. coli* MA1935 *icd* mutant	*E. coli* BL21 pJE6
Glutamate auxotrophy	-	positive	negative
Growth rate	0.74 ± 0.04	~ 2.2. fold decrease	No change
Glucose consumption rate	7.0 ± 0.72	~ 1.75 fold decrease	~ 1.5 fold decrease
Glucose consumed	45.5 ± 4.0	~ 2 fold decrease	~ 1.2 fold decrease
G-6-PDH	0.2 ± 0.03 U	~ 1.5 fold decrease	No change
CS	0.08 ± 0.006 U	No change	~ 1.2 fold increase
ICDH	1.01 ± 0.09 U	ND	~ 3-4 fold decrease
PPC	0.05 ± 0.007	No change	-
ICL	0.007 ± 0.002	No change	No change
Citrate (Intracellular)	2.57 ± 0.58 mM	~ 1.5 fold increase	~ 2 fold increase
Acetate	20 ± 3 mM	ND	~ 1.5 fold decrease

Table 13.4: Effect to *icd* mutation and P_{tac} as-*icd* (pJE6) in *E. coli* BL21 on glucose.

E. coli MA1935 *icd* mutant was reported to accumulate as high as 11mM citrate on rich medium (Aoshima, et at., 2003) (**Table 13.4**). In contrast to *E. coli* MA1935, as-*icd* expression enabled growth on glucose even in the absence of glutamate. *E. coli* MA1935 *icd* mutant showed poor growth rate, glucose consumption rate and reduced glucose consumed whereas as-*icd* expression had similar effects on glucose consumption rate and glucose consumption but did not alter the growth rate. Mutation of *icd* gene decreased G-6-PDH activity by ~ 2 fold decrease supporting the poor growth rate but did not alter CS activity which was contradictory to the ~ 2 fold increased CS activity monitored in *E. coli* BL21 under as-*icd* expression. Both, *E. coli* MA1935 and *E. coli* BL21 expressing as-*icd* demonstrated high citrate accumulation and reduced acetate

secretion suggesting that the partial block in *icd* gene achieved using antisense benefited growth and increased citrate accumulation.

E. coli MA1935 *icd* mutant demonstrated low citrate accumulation on minimal medium as compared to that reported for rich medium. This may be attributed to the limiting OAA levels or CS enzyme activity. Hence, *S. elongates* PCC 6301 *ppc* gene and *E. coli cs* gene were overexpressed independently and in combination in *E. coli icd* mutant (Chapter 8, 9 and 10, **Table 13.5**). ~ 3 fold increased PPC activity improved biomass without altering growth and glucose consumption rate. Increased PPC activity decreased CS activity and citrate accumulation suggesting the flux was higher towards anaplerotic pathway.

Parameters	*E. coli* MA1935 *ppc* overexpression	*E. coli* MA1935 *gltA* overexpression	*E. coli* MA1935 *ppc* and *gltA* overexpression
Growth rate	High	unaltered	High
Glucose consumption rate	Unaltered	~ 2 fold high	~ 2 fold decrease
Glucose consumed	unaltered	reduced	Reduced
Biomass	~ 1.5 fold increase	~ 5 fold increase	~ 3 fold increase
G-6-PDH	unaltered	unaltered	~ 3 fold increase
CS	~ 2 fold decrease	~ 2 fold increase	~ 4 fold increase
PPC	~ 3 fold increase	unaltered	~2.5 fold increase
ICL	~ 3 increase	unaltered	~ 4 fold increase
Citrate (intra cellular)	~ 4 fold decrease	~ 5 fold increase	~ 2.5 fold increase
Citrate (extra cellular)	ND	60 ± 5 μM	26 ± 3 μM
Acetate	ND	~ 4 fold increase	~ 8 fold increase

Table 13.5: Effect of *ppc* and *cs* gene overexpression in *E. coli* MA1935 *icd* mutant on glucose.

Overexpression of *gltA* gene by ~ 2 fold increased glucose consumption rate, biomass, citrate accumulation, secretion and acetate secretion. On the other hand, co-expression of *ppc* (~2.5 fold) and *gltA* (~ 4 fold) in *E. coli* MA1935 *icd* mutant decreased citrate accumulation and secretion compared to the citrate levels obtained upon *gltA* overexpression. Increase in acetate secretion was found in both conditions. Earlier

reports demonstrated that PPC directs the flux toward anaplerosis (Farmer and Liao, 1999) and *gltA* overexpression improved biomass and reduced acetate secretion (De Maeseneire et al., 2006). In *icd* mutant, *ppc* overexpression improved biomass with significant increase in ICL activity but decreased citrate accumulation and acetate secretion. On the contrary, *gltA* overexpression showed high citrate and acetate accumulation with unaltered ICL activity. Co-expression of *ppc* and *gltA* genes demonstrated effects similar to that of *ppc* overexpression excepting high levels of acetate secretion.

Parameters	*E. coli* DH5α pJE2 : pVSD1	*E. coli* BL21 pJE2:pVSD1
Growth rate	No change	No change
Glucose consumption rate	No change	No change
Glucose consumed	No change	No change
Biomass	~ 1.2 fold increase	~ 2 fold increase
G-6-PDH	~ 1.5 fold decrease	No change
CS	~ 2 fold increase	~ 1.8 fold increase
ICDH	No change	No change
ICL	ND	No change
Citrate (Intracellular)	~ 1.5 fold increase	~ 2 fold increase
Acetate	~ 2.25 fold decrease	~ 2 fold increase

Table 13.6: Effect of *gltA* overexpression in *E. coli* DH5α and *E. coli* BL21 on glucose.

Overexpression of *gltA* (~ 2 fold) gene showed similar effects on the growth, glucose consumption rate, and biomass and citrate accumulation in both the *E. coli* strains. But *E. coli* DH5α showed reduced G-6-PDH activity and acetate secretion compared to *E. coli* BL21 (**Table 13.6**).

Overexpression of *gltA* coupled with decreased ICDH activity in *E. coli* DH5α improved growth rate and biomass but decreased glucose consumption rate compared to *E. coli* DH5α expressing as-*icd* alone. Co-expression of *gltA* and as-*icd*

genes improved citrate accumulation as well as acetate secretion which could be attributed to poor TCA flux.

Parameters	*E. coli* DH5α pVS2k3	*E. coli* DH5α pVS2k3g :pJE2: pVSD1
Growth rate	No change	~ 1.5 fold increase
Glucose consumption rate	~2fold increase	~ 1.2 fold decrease
Glucose consumed	No change	~ 1.5 fold increase
Biomass	No change	~ 2 fold increase
G-6-PDH	~ 1.2 fold decrease	No change
CS	~ 2 fold increase	~ 2.5 fold increase
ICDH	~3-4 fold decrease	~ 2 fold decrease
ICL	ND	ND
Citrate (Intracellular)	~ 2 fold increase	~ 4 fold increase
Acetate	~ 2 fold increase	~ 2 fold increase

Table 13.7: Effect of *gltA* overexpression and decreased ICDH activity in *E. coli* DH5α pVS2k3 on glucose.

Parameters	*E. coli* BL21 pJE6	*E. coli* BL21 pJE8:pJE2: pVSD1
Growth rate	No change	~ 2 fold increase
Glucose consumption rate	~ 1.5 fold decrease	~ 1.5 fold increase
Glucose consumed	~ 1.2 fold decrease	No change
Biomass	~ 2 fold decrease	~ 1.7 fold decrease
G-6-PDH	No change	No change
CS	~ 1.2 fold increase	~ 2.5 fold increase
ICDH	~ 3-4 fold decrease	~ 2.5 fold decrease
ICL	No change	No change
Citrate (Intracellular)	~ 2 fold increase	~ 3 fold increase
Acetate	~ 1.5 fold decrease	No change

Table 13.8: Effect of *gltA* overexpression and as-*icd* in *E. coli* BL21 on glucose.

Co-expression of *gltA* and as-*icd* genes in *E. coli* BL21 improved growth rate and glucose consumption rate with increased citrate accumulation which was contradictory to the results of *E. coli* BL21 expressing as-*icd* alone and *E. coli* DH5α with co-expression **(Table 13.7, 13.8 and 13.9)**.

In both the *E. coli* strains, co-expression of *gltA* with decreased ICDH activity increased citrate accumulation compared to the levels obtained with independent as-*icd* and *cs* overexpression but varied with respect to acetate secretion. Although increased CS activity increased citrate accumulation, co-expression of *gltA* and as-*icd* supported high citrate accumulation but the citrate levels were lower compared to *gltA* overexpression in *E. coli icd* mutant. This supported the view that citrate transport system is inefficient.

Glycerol is distinct from glucose as its transport is independent of PTS transport and the modifications at the anaplerotic node could affect PEP levels on glucose as PEP is required for transport of glucose. On glycerol, *E. coli* strains demonstrated poor growth and low acetate secretion compared to that observed on glucose **(Table 13.10)**. High CS activity and citrate levels were observed in *E. coli* DH5α supported high TCA flux even though enzyme activity does not always correlate with flux. On the contrary low citrate levels with high CS activity were observed in *E. coli* BL21.

Parameters	***E. coli* DH5α pVS2k3g :pJE2: pVSD1**	***E. coli* BL21 pJE8:pJE2: pVSD1**
Growth rate	~ 1.5 fold increase	~ 2 fold increase
Glucose consumption rate	~ 1.2 fold decrease	~ 1.5 fold increase
Glucose consumed	~ 1.5 fold increase	No change
Biomass	~ 2 fold increase	~ 1.7 fold decrease
G-6-PDH	No change	No change
CS	~ 2.5 fold increase	~ 2.5 fold increase
ICDH	~ 2 fold decrease	~ 2.5 fold decrease
ICL	ND	No change
Citrate (Intracellular)	~ 2 fold increase	~ 3 fold increase
Acetate	~ 2 fold increase	No change

Table 13.9: Effect of *gltA* overexpression and as-*icd* in *E. coli* DH5α and *E. coli* BL21 on glucose.

Parameters	*E. coli* DH5α	*E. coli* BL21
Growth rate	0.13 ± 0.015	0.34 ± 0.02
Glycerol consumed	78 ± 5.29	45.8 ± 3.6
G-6-PDH	0.096 ± 0.003 U	0.12 ± 0.03 U
CS	0.09 ± 0.005 U	0.09 ± 0.005 U
ICDH	0.3 ± 0.04 U	0.6 ± 0.05 U
ICL	ND	0.003 ± 0.00 U
Citrate (Intracellular)	3.12 ± 0.14	0.25 ± 0. 05
Acetate	5.7 ± 0.4	ND

Table 13.10: Physiological and metabolic differences between *E. coli* DH5α and *E. coli* BL21 on glucose.

Parameters	*E. coli* MA1935 *ppc* overexpression	*E. coli* MA1935 *gltA* overexpression	*E. coli* MA1935 *ppc* and *gltA* overexpression
Growth rate	No change	No change	~ 2 fold increase
Glycerol consumed	No change	~ 2 fold increase	~ 2 fold increase
G-6-PDH	No change	~1.5 fold increase	~ 1.2 fold increase
CS	~1.7 fold decrease	~ 2 fold increase	~ fold increase
PPC	~ 3fold increase	No change	~ 2.5 fold increase
ICL	~1.5 fold decrease	No change	~ 3 fold increase
Citrate (intra cellular)	~ 2 fold decrease	~ 11 fold increase	~ 4 fold decrease
Acetate	ND	~1.8 fold increase	~ 4.4 fold increase

Table 13.11: Effect to *ppc* and *gltA* gene overexpression in *E. coli* MA1935 *icd* mutant on glycerol.

P_{fruB} as-*icd* overexpression in *E. coli* DH5α on glycerol demonstrated no change in the ICDH activity supporting that the promoter was regulated by the levels of fructose 1-6-bisphosphate which were lower on glycerol. Modulations at the anaplerotic node

involving *ppc* and *cs* overexpression both independent and together demonstrated higher citrate accumulation compared to citrate levels obtained on glucose.

Hence, the present study demonstrates that decreased ICDH activity coupled with overexpression of *gltA* gene enhanced citrate accumulation in both the *E. coli* strains but differed with respect to growth rate, glucose consumption rate and regulation of some of the enzyme activities.