# CHAPTER 6

# METABOLIC ADVANTAGE AND ENERGY ECONOMY OF CHOLINE LACTATE IN BACTERIAL GROWTH MEDIA

## 6.1 INTRODUCTION

It is well known that a growth medium is prepared to support the growth of microorganisms. It would be greatly beneficial to design a media that is effective and efficient in terms of the nutritional and energy needs of the growing bacterial colony. Substances that are capable of multiple biochemical pathways involving anabolic and catabolic fates in a manner that will facilitate rapid bacterial growth machinery, without wasteful energy-dissipating fates, may be suitable substrates for an efficient growth media. In the present study, the choice of efficient media was tested for cultivating the halotolerant bacterium namely, S. lentus strain, isolated from marine sea water. The selection of this halotolerant strain is based on the requirement for the bioremediation of industrial effluents, such as effluents with high salinity and mixed industrial waste waters, especially effluents from the leather manufacturing industry. Halotolerant strains are also efficient in degrading xenobiotic compounds that result from anaerobic degradation of azo dyes.

Here, some key experimental findings and analyses on the growth of S. lentus cultured in two different media: are present (i) Glucose, (ii) Choline lactate. Choline lactate (CL), an ionic liquid, has eight carbons and a nitrogen atom, while glucose has six carbon atoms. Glucose is poised for complete oxidation under aerobic conditions, as $6CO_2$ per mole and energy release, while the ionic salt, choline lactate, follows a distinct metabolic trajectory that is analyzed in this chapter.

Ionic liquids are molecularly flexible through different cations and anions combinations and allow fine tuning of the physical properties especially substrate product partitioning and biocompatibility (Gangu et al 2009). Two important factors that are considered for any biotechnological process to be environmentally friendly ('green') are: (i) the nutrient salt, used to increase the populations of bacteria, should not be toxic to the environment, and (ii) the biomass generated at the end of the process should not contaminate the environment. Choline lactate is a suitable candidate in this regard, choline salts are easily biodegradable. Recently, the enhanced solubility and stability of biological proteins and DNA in novel biocompatible ionic liquids based on choline cation and anions such as dihydrogen phosphate (Vijayaraghavan et al 2010) was reported. Although the metabolism of choline (Meck et al 2003) and lactate (Gladden et al 2004) have been individually studied in microbial system, the metabolic utilization of choline lactate as an ionic liquid medium is not yet reported.

One of the chief advantages of knowing the metabolic pathways apriori is to predict the maximal yields for the biosynthesis of valuable products (Famili et al 2003) and (Mavrovouniotis et al 1993). The fascinating subject of metabolic engineering is devoted to understanding the cellular pathways for biochemical transformations, energy transduction and supramolecular assembly. Metabolic pathways and the cell biochemistry continue to be a subject of intense research activity in order to explore the pathways of carbon and nitrogen metabolism that provide the building blocks biomacromolecular synthesis and growth. Recently, the network topology of glucose metabolism and it's in vivo operation was successfully attempted by employing GC-MS (Fischer et al 2003) and $^{13}C$ tracer experiments on seven bacterial species (Fuhrer et al 2005) .

The central focus of the present research was to monitor and analyze the differential fates of the cationic and anionic constituents of the ionic salt, choline lactate (Carbon and Nitrogen source) in growing S. lentus. This has been achieved by a combination approach using biocalorimetry and spectroscopy. The metabolic and energy aspects of choline lactate have been compared with the conventional C-source, glucose.

## 6.2 MATERIALS AND METHODS

The details about the chemicals and reagents, halotolerant bacterial strain and media employed here were the same as in previous chapters 4 and 5.

### 6.2.1 FT-IR and $^{13}C$ NMR Analysis

FT-IR analysis of the degraded samples was carried out using ABB MB3000 FT-IR Spectrometer. The culture medium containing the degradation products was centrifuged and the 5µl of supernatant was sandwiched between two plates of high purity salts like Potassium Bromide (KBr). This sample was placed in the Infrared Spectrum beams and the spectrum recorded.

The supernatant was concentrated in a Rotary Evaporator (Buchi) as the samples for NMR analysis required a concentrated sample unlike FT-IR studies. All NMR experiments were made on a JEOL spectrometer operating at 500 MHz (ECA-500). For $^{13}C$ NMR (at 125 MHz) chemical shift studies, TSS (3-(trimethyl silyl) propionic 2,2,3,3-$d_4$ acid, sodium salt) in $D_2O$ was used as an internal reference. The $^{1}H$ and $^{13}C$ chemical shifts are referred with respect to methyl carbon of TSS, respectively, which was arbitrarily set as 0 ppm.

## 6.3 RESULTS AND DISCUSSION

### 6.3.1 Growth of S. lentus in Choline Lactate Media

Figure 5.5 presents the relative colony densities of S. lentus grown with glucose and choline lactate in nutrient agar. The choline lactate plate clearly shows greater bacterial population. The growth rate of the strain S. lentus, with choline lactate (2.0 g/l), and in glucose media (5 g/l) as growth media was then compared. The thermal responses of the metabolic activity with glucose as a carbon source for growing S.lentus is presented in Chapter 4. Glucose was found to be growth limiting at a concentration of 5 g/l, while choline lactate showed limiting concentration of 2 g/l in this study. It is thus observed that a lesser concentration of choline lactate is sufficient to achieve similar growth rate as in glucose at its 5 g/l concentration shown in Figure 6.1.

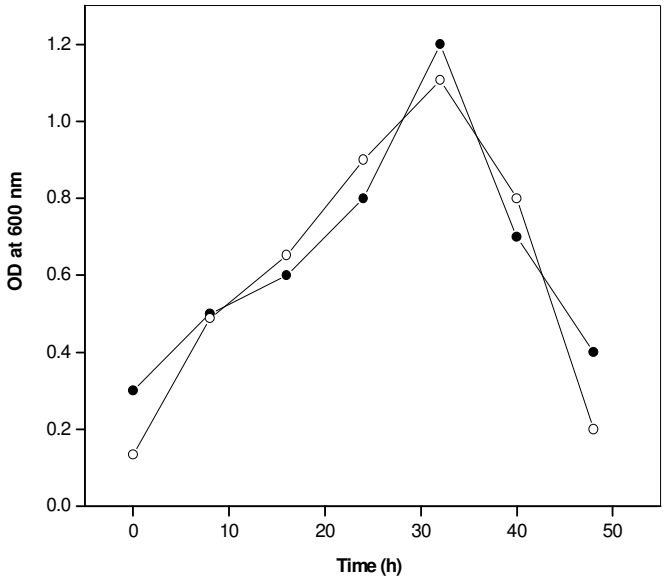


**Figure 6.1 Growth patterns of S. lentus in MS media in presence: (Glucose 5 g/L (●), Choline lactate 2 g/L (○))**

Choline lactate concentration of 2 g/L was selected for further metabolic and thermodynamic evaluations, as S. lentus showed maximum growth rate at this concentration.

### 6.3.2 Study of Metabolic Pathways Involved in Choline Lactate Consumption by S. lentus using $^{13}C$ NMR Spectroscopy

$^{13}C$ NMR of the samples withdrawn at 4, 12, and 40$^{th}$ h during the growth metabolism of S. lentus promoted by choline lactate as carbon source are presented in Figure 6.2. The 0$^{th}$ h sample did not show appreciable changes in the structure of the choline salt. The methyl and methine groups of the lactate anion appears to be the most preferred and easily utilizable carbon sources in comparison to methyl and methine groups of the choline. The $^{13}C$ NMR of 4$^{th}$ h sample indicates disappearance of signals at 20.45 ppm which are characteristic of methyl and methine ($CH\text{-}CH_3$) carbons of the lactate anion. The $^{13}C$ NMR also shows the appearance of additional peak at 160.32 ppm, which is characteristic for the presence of the carboxylic acid. The disappearance of the methyl and methine carbon signal at 20.45 ppm of the lactate anion is a clear indication of its utilization by the organism S. lentus during the initial phases of its growth and multiplication.

The sample collected at the 12$^{th}$ h during the metabolism of S. lentus is given in Figure 6.2. The spectrum shows disappearance of peaks at 20.45, 182, and 192 ppm which is due to methyl and methine, COO of lactate. The appearance of additional signals, as in the case of 4$^{th}$ h sample, at 160.33, 160.56 and 160.64 ppm respectively, is due to the formation of carboxylic acids. In this spectrum, very interestingly the number of signals in the region of 50 to 60 ppm has been reduced. It may be noted that the signals in the region 50 to 60 ppm were due to the methyl carbons of the choline cation. Here, the reduction in the number of signals characteristic of methyl carbons of choline cation is a clear indication in the shift of the utilization of methyl carbon of choline on exhausting the methine and methyl carbons of lactate anion.

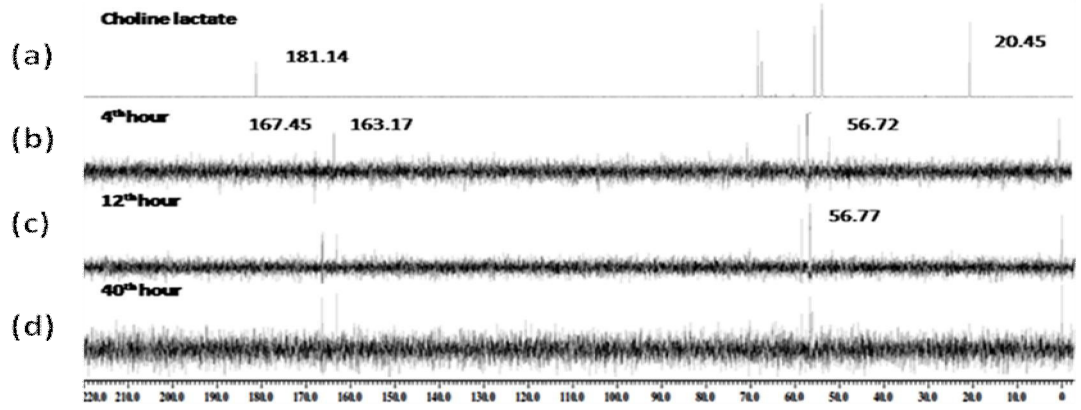


**Figure 6.2** **$^{13}$C NMR showing the progressive utilization of choline lactate during growth metabolism of S. lentus: ((a) 0$^{th}$ h (b) 4$^{th}$ h, (c) 12$^{th}$ h, and (d) 40$^{th}$ h)**

The 40$^{th}$ h sample collected during the growth metabolism promoted by the choline lactate was also characterized for $^{13}$C NMR (Figure 6.2). The spectrum shows signals similar to that of 12$^{th}$ hour sample, excepting that signal intensity and the number of peaks in the region 50 to 60 ppm were low. The 40$^{th}$ h sample also leads us to conclude that the microorganism preferentially utilizes the carbons in the lactate anion, and when the lactate is exhausted, it shifts to carbons of the choline cation progressively and slowly. In a recent study, it was shown that choline consumption proceeded slower than that of lactate (Deive et al 2011). There are numerous studies indicating that choline is biocompatible, and the degree to which it is biodegradable varies with different kinds of microbes, and their environment (Boethling et al 2007). In a study of 123 microbial strains (Schisler et al 2006), differential degradation of choline was noted, where choline was found to be intracellularly accumulated and oxidized to glycine betaine to serve as an Osmoprotectant in osmotically stressed E. coli. The enzymatic catabolism of choline to glycine and serine amino acids has been described in other reports that will be discussed in the following sections. Therefore, the ways in which lactate and choline are metabolized

independently in various microbial systems differ from the metabolism of choline-lactate ionic salt. Even if it is assumed that choline lactate in the growth medium dissociated in to lactate and choline ions, the spectroscopic data presented here clearly suggest that their simultaneous utilization is not taking place.

FTIR studies of the samples collected during the growth metabolism are presented for the 40$^{th}$ h sample alone in Figure 6.3 Unlike the $^{13}$C NMR studies, the spectral resolution of infrared spectrums for 0, 4 and 12$^{th}$ h samples were hampered by the presence of residual moisture, which was difficult to separate from the sample. As a result, the adsorption intensity in the region 3300 – 3500 cm$^{-1}$ is high, leading to masking of the other signals in the spectrum.

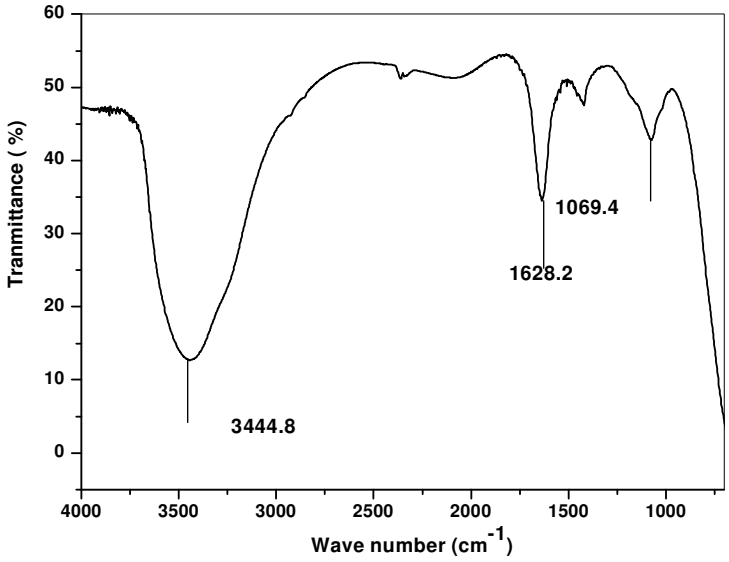


**Figure 6.3 FTIR analysis of 40$^{th}$ h sample**

The 40$^{th}$ h sample presented in Figure 6.3 shows two characteristic stretching vibrations at 3448.3 and 1628.2 cm$^{-1}$, indicating the presence of N-

H and C=O groups in the sample. Due to structural similarities between choline salt and amino acids (aliphatic contents) the formation of urea as an end product is proposed for the growth metabolism of S. lentus promoted by choline lactate as a carbon source. The FT-IR spectra of the 40$^{th}$ h sample were independently compared with the FTIR spectra of urea sample (Appendix 2) from NIST Chemistry (NIST Chemistry Web Book).

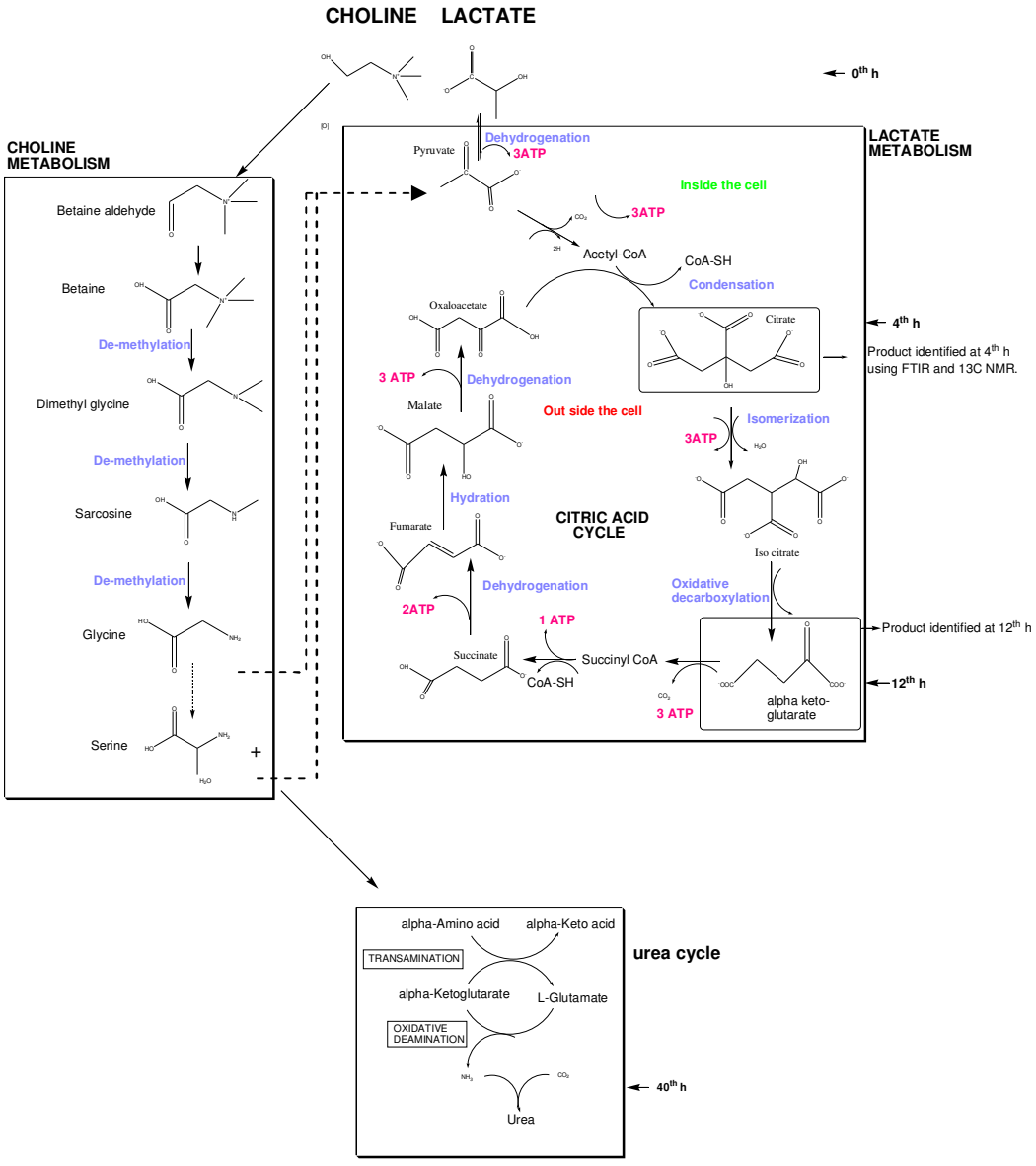


**Figure 6.4 Classical subdivision of choline lactate metabolism by S. lentus**

Based on the above studies, the metabolic pathway for the consumption of choline lactate by the organism S. lentus was worked out, and has been illustrated using Chem Draw 8.0 in Figure 6.4.

### 6.3.3 Biochemical Advantage of Choline Lactate Growth Media

Choline lactate as a nutrient media is an interesting molecule from the biochemical stand-point, in that choline and lactate endow their distinct metabolic capabilities that synergize and enhance the bacterial growth rate in a much more efficient manner than the glucose C-substrate.

Duplication of genetic and structural materials precedes cell division. For all these growth and anabolic activities, the cells require energy in a usable form, such as ATP (adenosine triphosphate, the energy-currency of the cell). From the experimental analysis, it is apparent that the lactate unit of choline lactate in the media was consumed entirely releasing $CO_2$, $H_2O$, and energy, following the TCA cycle. The choline moiety of choline lactate is capable of several fates depending upon the metabolic state and environment of the growing bacterial cells. Choline may be transformed into amino acids, such as glycine and serine. These may further lend themselves to transamination reactions leading to formation of other amino acids. Amino acids may be used for the synthesis of proteins and nucleic acid nitrogenous bases in the growing bacteria, or may be used for gluconeogenesis or lipogenesis. Further, we have recorded the formation of urea at the end of 40 hours of incubation in choline-lactate media, that indicates the salvaging of nitrogen into the carbonyl diamide, urea.

Choline is an important component of cell wall teichoic acids, making this amino alcohol an important nutrient for the growth of many bacteria. Teichoic acids are complex anionic polymers abundant in the cell wall of the gram-positive bacteria, and in lesser quantities in the cell

membrane. In a specific study on Streptococcus oralis grown in choline-media, about 80 to 90% of choline was reported to be incorporated into the cell wall teichoic acid, and 10% in the cell membrane (Horne et al 1993). Their report also indicated that cells grown in choline-free medium showed slow growth rates, had cell walls with very less phosphate and no choline, and abnormal shape and size of cells, which were reversed by addition of choline to the media (Horne et al 1993).

Tomasz et al (1968) had pointed out that in pneumococci, the choline residues in the teichoic acid of the cell envelope were not only needed for bacterial growth, but also for facilitating cell division and allowing the physical separation of daughter cells from one another, by incorporating new choline molecules on the cell surface at a distinct growing zone in the equatorial region. In the light of this observation, and related findings in other bacteria, it was suggested that some structural and/or functional features of the cell division apparatus of bacteria was utilized for the uptake of DNA molecules in genetic transformations (Tomasz et al 1971)

Choline is a component of the important membrane phospholipid, phosphatidylcholine (lecithin), found mainly in eukaryotic membranes, and in few prokaryotes. A large number of bacteria, involved in symbiotic or pathogenic interactions with plant or animal hosts, contain phosphatidylcholine in their membranes, where they source the choline nutrient from the plant or animal hosts. In bacteria, phosphatidylcholine is synthesized by enzymatic methylation of phosphatidylethanolamine (catalysed by phospholipid N-methyltransferase), or directly from choline and CDP-diacylglycerol by phosphatidylcholine synthase enzyme, as in the mutants of the microsymbiotic soil bacterium Sinorhizobium (Rhizobium) meliloti (defective in phospholipid N-methyltransferase) (De Rudder et al 1999) .

The choline oxidative catabolism results in the formation of glycine betaine, followed by progressive demethylation to form dimethyl glycine, sarcosine, and then glycine (Smith et al 1988). The betaines produced as intermediates here are significant to bacterial survival in several ways. Betaines are a methyl donor that is vital for several biochemical reactions. Microorganisms also utilize betaines as organic Osmoprotectant, allowing water retention in cells, thus protecting from themselves from osmotic stress, drought, high salinity, or high temperature (Landfald et al 1986). The enzyme activity, protein structure and membrane integrity are thus protected. Smith et al (1988) showed the osmotic effects on the biosynthesis and degradation of glycine betaine from choline, where it was suggested that the function of glycine betaine as a carbon and nitrogen nutritional source, or as an Osmoprotectant, was determined by the osmolarity of the growth medium.

### 6.3.4 Correlating Metabolic Pathway and Heat Release

In the metabolic pathway proposed in Figure.6.4, citric acid cycle produces energy by oxidatively breaking down lactate. However, the biosynthesis of urea from the choline moiety of the ionic salt consumes energy. A total of 18 ATP are generated via TCA cycle inside the cell in the steps outlined in Figure 6.4. The carbon of the lactate is completely metabolized to $CO_2$ along with the ATP generation. The metabolic heat release profiles presented in Figure 5.11 can be explained clearly based on the proposed metabolism. At the stage of citric acid formation after the $4^{th}$ h, six ATPs would be formed. This is consistent with the first major peak in the heat profile. Following this peak, a short decline in the heat profile was observed, which can be attributed to the lack of any energy yielding steps until the formation of α- keto glutarate at the end of $12^{th}$ h. This is followed by a sharp increase in the heat release profile that doubles the earlier peak, which may be explained by the greater number of energy yielding steps

resulting in 12 molecules of ATP released between the formation of α- keto glutarate and replenishing of oxaloacetate in the TCA cycle. This metabolic stage coincides with the second phase of exponential growth of S. lentus. On the other hand, the choline cation (carbon-nitrogen source) consumption may network with the protein, lipid and/or nucleic acid metabolism. The accumulation of urea in the $40^{th}$ h sample could be one of the fates of the choline-associated nitrogen. Three ATP molecules are consumed during urea biosynthesis. In addition to the metabolic pathways outlined here, several other concurrent reactions may be undergone by the intermediates of TCA cycle as well as choline metabolism. These reactions may explain the peaks recorded after $26^{th}$ h in Figure 5.11.

### 6.3.5 Delineation of Choline Cation and Lactate Anion Metabolic Pathways in S. lentus Grown in Choline-lactate Ionic Salt Media

The $^{13}C$ NMR and FT-IR analyses revealed the differential utilization of choline and lactate constituents of the ionic salt, choline lactate by S. lentus. Although lactate is gluconeogenic, the preferred pathway of lactate utilization seems to be driven by the energy needs of the growing bacterial colony. $^{13}C$ NMR and FT-IR studies conclusively revealed that the carbon atoms of the lactate ion were consumed during early stages of the growth of S. lentus. The choline cation is targeted by the organism after complete oxidation of lactate. Based on the $^{13}C$ NMR and FT-IR studies, metabolism of choline lactate is presented in Figure 6.4. Two pathways may be operational which will account for the consumption of choline lactate: (i) Lactate is oxidatively broken down via TCA cycle (citric acid cycle) to yield $CO_2$, $H_2O$ and energy; (ii) Choline cation is likely to follow reactions leading to the formation of glycine, and enter into intermediary metabolism. Consistent with the typical fate of nitrogen, we observed the accumulation of urea that could be attributed to the choline in the ionic salt media.

Initially, the lactate oxidizes to keto carboxylic acid. The pyruvate so formed is oxidatively decarboxylated to a 2-carbon intermediate, acetyl CoA. Acetyl CoA now reacts with oxaloacetate inside the cell to form citric acid. This was identified at $4^{th}$ h sample through FT-IR and $^{13}C$ NMR studies. After subsequent reactions in the TCA cycle, the five-carbon molecule α-ketoglutarate was obtained. This intermediate was identified at the end of $12^{th}$ h sample. It is the precursor for succinate, a four-carbon intermediate. Succinate undergoes further hydration and redox reactions to ultimately recycle the oxaloacetate molecule. At the end of this turn of TCA cycle, lactate of the ionic salt gets metabolized completely discharging its three carbons as carbon dioxide, water, and energy.

In the present analysis, the diamide obtained in the $40^{th}$ h sample, is suggested to be urea. The FT-IR spectrum of $40^{th}$ h sample is quite characteristic of the urea molecule. The biosynthetic source for the diamide formation may be due to the nitrogen metabolism of choline, through the metabolic fates of choline leading to the formation of amino acids, glycine and serine (Smith et al 1988 and Lisa et al 2007).

Considering the thermodynamic data obtained here, and the nature of the biochemical products accumulated, it can be said that choline lactate follows the metabolic pathway typical of complete oxidative breakdown of lactate and partial utilization of choline cation as shown in Figure 6.4.

### 6.3.6 Comparison of Theoretical and Experimental Reaction Enthalpy

The theoretical estimation of heats of reaction for the proposed metabolic pathway was carried out using the ASTM Software CHETAH (Shanley et al 1995). It was found to be 455 kJ/mol. This value is comparable with the experimentally derived value of 435 kJ/mol that has already been calculated.

### 6.3.7 Comparative Energetics

Biothermodynamic analysis is complex owing to the inherent complexity of living cells, characterized by numerous biomacromolecules, the tightly regulated metabolic pathways and redox mechanisms, and heterogeneity of phases. The driving energy for growth is synchronized suitably by biosynthetic anabolic reactions and the energy-releasing catabolic reactions, to achieve a balance between pace of growth and biomass (VonStockar et al 2010). Here, it will be apt to mention that the biological system is a coherent array of chemo-dynamical molecular machines, and not a heat engine, as expressed by Welch (1993), wherein much of the observed heat-production is due to the "cost" of maintaining the non-equilibrium energy-states (redox bioenergetics), which is required for the intermediary metabolism to sustain life and growth. Further, it was suggested that in cellular physiology, speed of metabolic reactions dominates over efficiency and that in a continually evolving living system, efficiency should be considered relative to the physiological, ecological, and evolutionary context (Welch 1993).

In the present study, the thermodynamic profiles of the metabolites observed at different time intervals in choline lacate versus glucose-grown bacteria have been compared (Figure 5.12). The power versus time graph clearly indicates the bacterial requirement of energy from the system during the initial phase in glucose-based medium. In sharp contrast, the choline lactate based medium requires the bacteria to absorb negligible threshold energy, beyond which the bacterial cells sustain their growth utilizing the energy from intermediary metabolism. This is because the growing cell utilizes the three-carbon lactate anion as the immediate energy currency through the TCA cycle in comparison with the longer time taken to withdraw energy from the six-carbon glucose. It is also noted that the energy associated with choline lactate supported growth is higher than the glucose grown cells.

This may be explained by the possibility of choline catabolites entering into intermediary metabolism involving TCA cycle (via formation of glycine and serine, (Smith et al 1988).

The correlation between the biomass and energetics of growth corroborates with the report by (VonStockar et al 2010). Here, we observe the biomass in the choline lactate grown cells to be relatively lesser by 0.003 g/l than the glucose counterpart (Figure 6.5). On the same note, the bacterial growth in nutrient agar with choline lactate (Figure 5.5), and the energy (Figure 5.12) associated with the choline lactate in MS media grown cells were higher than in the glucose-based medium. Thus, the shift of balance that promotes cell growth at the expense of biomass is favorably achieved in choline lactate media.

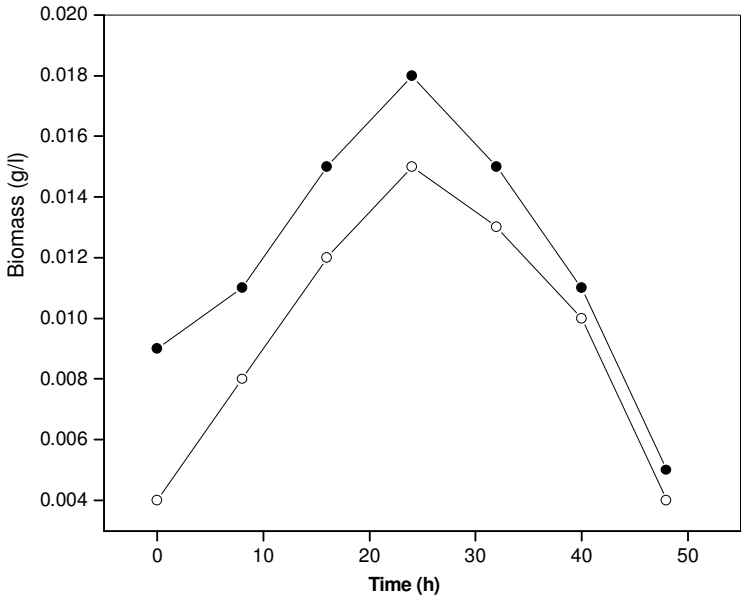


**Figure 6.5 Biomass patterns of S. lentus in MS media in presence: (Glucose 5 g/l (●), Choline lactate 2 g/l (○)).**

## 6.4 CONCLUSIONS

- A lesser concentration of choline lactate is sufficient to achieve similar growth rate of S. lentus as in glucose.
- The ways in which lactate and choline are metabolized independently in various microbial systems differ from the metabolism of choline ionic salt.
- The biomass in the choline lactate growth cells is found to be relatively larger than the glucose counterpart.
- The spectroscopic data suggested that the utilization of choline and lactate are not simultaneous processes.
- The metabolic pathway for the consumption of choline lactate by S. lentus has been worked out.
- The heat increase pattern could be well correlated to the metabolic pathway of choline lactate consumption.
- Choline lactate follows the metabolic pathway typical of complete oxidative breakdown of lactate anion and partial utilization of choline cation.
- The experimental analysis seem to suggest that choline lactate as (Carbon and nitrogen) source for growing S. lentus offers a distinct biochemical and energetic advantage relative to traditional C source.
- This may be particularly attractive in the industrial setting where materials and energy efficiency are of paramount significance.