

Over Production of Lactate in *E.coli* Engineered With an Acrylate Pathway

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Abstract: Lactic acid is widely used in the food, cosmetic, pharmaceutical, and chemical industries and has received increased attention for use as a monomer for the production of bio-degradable polylactic acid. It can be produced by either biotechnological fermentation or chemical synthesis, but the former route has received considerable interest recently, due to environmental concerns and the limited nature of petrochemical feed stocks. The recombinant *E. coli* VK-23 encoding the enzymes of acrylate pathway (lactoyl-CoA dehydratase, acryloyl-CoA reductase and propionyl-CoA transferase) has been demonstrated earlier for overproduction of lactic acid. In this study we intend to find out the enzymes responsible for lactate overproduction and to improve the lactate productivity levels by using fed-batch bioreactor. A maximum productivity level of up to 24.2 g l^{-1} (% of yield 96%) in 14 hours was obtained with maximum specific and volumetric productivity of $0.8 \text{ g g}^{-1} \text{ h}^{-1}$ and $2.4 \text{ g l}^{-1} \text{ h}^{-1}$ respectively. The productivity levels attained seemed to be comparatively similar with that of metabolically engineered lactic acid overproducers. This recombinant *E. coli* with a new acrylate pathway has great potential to meet the growing market demand of fermentation based lactic acid production due to high growth rate and tolerance to organic acids.

Keywords: Recombinant *E.Coli*, Lactic acid, Acrylate pathway, Fed batch, Volumetric productivity

I. INTRODUCTION

Lactic acid has a long history of uses for fermentation and preservation of human food stuffs. It was first discovered in sour milk by Scheele in 1780. In early 1960s, a method to synthesize lactic acid chemically was developed due to the need for heat stable lactic acid in baking industry [1]. Lactic acid is classified as GRAS (generally recognized as safe) for use as food additive by US FDA (Food And Drug Administration). The chemical synthesis using petro chemical resources always results in racemic DL- lactic acid, whereas, an optically pure L(+) or D (-) lactic acid can be obtained by microbial fermentation when the appropriate micro-organism that can produce only one of the isomers is selected [2]. The optical purity of lactic acid is crucial to the physical properties of poly lactic acid and an optically pure lactic acid can be polymerized to a highly crystalline polylactic acid that is suitable for commercial uses [3]. Lactic acid is now considered to be one of the most useful chemicals, applied in food industry as a preservative, acidulant and flavouring, in the textile and pharmaceutical industries, and in chemical industry as a raw material for the production of lactate ester, propylene glycol, 2,3-pentanedione, propanoic acid, acrylic acid, acetaldehyde and dilactide [4]. Recently lactic acid consumption has increased considerably because of its role as a monomer in production of biodegradable polylactic acid, which is well-known as a sustainable bioplastic material. The worldwide demand of lactic acid estimated roughly to be 130000 to 150000 (metric) tons per year [5]. However, global consumption of lactic acid is expected to increase rapidly in near future. Since lactic acid is classified as GRAS for use as a food additive by the US FDA, it is widely used in every segment of the food

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industry where it serves in a wide range of functions such as flavouring, pH regulation, improved microbial quality, and mineral fortification. Moreover, it is used commercially in processed meat and poultry industries to provide products with an increased shelf life, enhanced flavour and better control of food borne pathogens. Lactic acid offers natural ingredients for cosmetic applications. Although primarily used as moisturizers and pH regulators, they possess multiple other properties such as antimicrobial activity, skin lightening and skin hydration. Since they are natural ingredients of human body, lactic acid and its salt fit perfectly to modern trend towards natural and safer formulation, and they produce such effects as skin lightening and rejuvenation, which make them very useful as active ingredients in cosmetics. Lactic acid is also used in the pharma industry as an electrolyte in many parenteral solutions that are intended to replenish the body fluids or electrolytes. Moreover lactic acid is used in wide variety of mineral preparations which include tablets prostheses, surgical sutures and controlled drug delivery systems. Main bottlenecks of chemical synthesis of lactic acid are limited petrochemical resources, generation of only racemic mixture and environmental issues[6]. So its use is restricted. To overcome these limitations research is shifting towards biotechnological processes for lactic acid production, which uses renewable sources as substrate, produce pure isomer of lactic acid and it is environmental friendly. Microorganism that can produce lactic acid can be divided in to two groups : bacteria and fungi. Most investigations of lactic acid production were carried out with lactic acid bacteria(LAB), Filamentous fungi, such as *Rhizopus*, utilize glucose aerobically to produce lactic acid[7]. Fungal fermentation has some advantages in that *R. Oryzae* requires only a simple medium and produces L (+) lactic acid, but it also require vigorous aeration because it is an obligate aerobes. In fungal fermentation, the low production rate, below 3g/(L.h), is probably due to the low reaction rate caused by mass transfer limitation. The lower product yield from fungal fermentation is attributed partially to formation of by products, such as fumaric acid and ethanol[8]. Only the homo fermentative LAB are available for commercial production of lactic acid [9]. Although there have been persistent attempts to produce lactic acid through fungal fermentation, LAB have been commonly used for production of lactic acid due to aforementioned disadvantages of fungal fermentation. *Escherichia coli* has many advantageous characteristics as a production host, such as rapid growth under aerobic and anaerobic conditions and simple nutritional requirements. Moreover well established protocol for genetic manipulation and a large physiological knowledge base should enable the development of *E.coli* as a host for production of optically pure D(-) or L(-) lactate by metabolic engineering. *E.coli*, a facultative anaerobe, carries out mixed acid fermentation of glucose in which the principal products are formate, acetate, D-lactate, succinate and ethanol[10]. Raw materials for lactic acid production should have the following characteristics: cheap, low levels of contaminants, rapid production rate, high yield, little or no by-product formation, ability to be fermented with little or no pre-treatment and year-round availability. Shengdeet *et al.*, constructed derivatives of *Escherichia coli* W3110 (prototrophic) as new biocatalysts for the production of D-lactic acid[11] Higher lactic acid concentrations may be obtained in batch and fed-batch cultures than in continuous cultures, whereas higher productivity may be achieved by the use of continuous cultures [12]. Our basic objective is to enhance lactic acid productivity by using bioprocess strategies.

II. MATERIALS AND METHODS

All chemicals, reagents and medium components used were of analytical grade. Standard reagents and medium components including glucose and inorganic salts were purchased either from Merck & Co (New Delhi, India) or Himedia Laboratories (Mumbai, India). Double distilled water was used in all experiments. Milli-Q water (18 ml) filtered twice through 0.45µm cellulose acetate membrane used for HPLC analysis (Sartorius, GmBH, Germany). *E. coli* Rosetta gami2(DE3), and *E. coli* VK23 (clone) a gram-negative rod shaped bacterium was used throughout the study. Stock cultures were maintained at -80°C in the LB media with 50% (v/v) glycerol. Dextrose obtained from Merck & Co (New Delhi, India) was used throughout the study. LB Media was used as composition consists of peptone (20gm/l), dextrose (20 gm/l) and yeast 10gm/l. Ampicillin concentration of 100mg/ml was prepared in distilled water, it was filter sterilised and stored at 4°C. Chloramphenicol concentration of 34 mg/ml was prepared in ethanol and stored at 4°C. Kanamycin concentration of 30 mg/ml was prepared in distilled water, it was filter sterilised and stored at 4°C. All media, unless otherwise stated, were

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sterilized in an autoclave at 121°C (15 lbs/inch² pressure) for 20 min. Trace elements and other heat-labile substances were filter sterilized through 0.2 micro metre cellulose acetate membrane filter (Sartorius, GmbH, Germany). Centrifugation was carried out using, Sigma bench-top centrifuge (USA) and Sorvall centrifuge with built-in refrigeration system (USA). The maximum liquid volumes of 1.5mL and 20-200mL respectively were centrifuged. Microscopic observation was performed using Nikon Eclipse 80i light microscope (Japan) with oil immersion lens. For fluorescence microscopy work, an Olympus BH-2 (Japan) microscope with fluorescence attachment was used. An excitation wavelength of approximately 460 nm was used for the study. Spectrophotometric analysis was performed using Hitachi U-1900 spectrophotometer. HPLC was performed on Shimadzu chromatograph equipped with Aminex HPX-87H column (300mm×7.8mm - BIORAD laboratories, USA) and Refractive Index detector. The amount of Carbon sources and metabolites like acetic acid, lactic acid, citric acid, succinic acid and ethanol were analyzed quantitatively during fermentation. The samples from the reactor were centrifuged at 8000 rpm for 15 min at 4°C to get clear supernatant, which was again filtered through 0.2µm filtration unit, finally 20µL of sample was injected into HPLC column. Mobile Phase = 5mM of Con. H₂SO₄, (i.e.) 272µL of Con. H₂SO₄ was added into 1-L of Milli Q. The log phase strain of *E. Coli* Rosetta gami2(DE3) was maintained in 25% of glycerol and stored at -20°C. The strain was also maintained on a LB agar Petri plate at 4°C. Sub-culturing was carried out periodically. The glycerol stock was used as a working stock. The screw cap vials were filled till the neck with the medium. 14 ml of LB broth supplemented with 1.5 ml of glucose (200g/l) was used. 10% of overnight grown culture was used to inoculate the media. Culture was kept in shaker at 200rpm and 37°C. When OD reached to 0.8-0.9 (3hrs) it was induced with 1mM IPTG and again kept in shaker at the same conditions for 6 hrs. 1.5ml of this culture was taken in eppendorf, centrifuged it and supernatant was collected for HPLC analysis. Two stage fed batch fermentation was carried out in New Brunswick bioreactor with working volume of 3 litres and initial concentration of 10g/l glucose. The culture at first was maintained aerobically (1vvm) to maximize the biomass (3g/l, 1OD₆₀₀ corresponds to 0.3g/l) and was subsequently shifted to anaerobic phase by flushing pure nitrogen (1vvm for 2 minutes) for acid production. Feeding was started at this point (10g/l glucose) to maintain minimum glucose level of 3g/l. At the same time it was then induced with 1mM IPTG. The culture was grown at 37°C at 600 rpm during aerobic phase to maintain 30% dissolved oxygen concentration (Y. Zhu *et al*-2006) and at 400 rpm throughout production phase. The pH was maintained at 7.0 by automatic addition of 5M NaOH. The fermentation was carried out for 14 hours after induction.

III. RESULTS AND DISCUSSION

The recombinant *E. coli* VK-23 encoding the enzymes of acrylate pathway (lactoyl-CoA dehydratase, acryloyl-CoA reductase and propionyl-CoA transferase) has been demonstrated earlier for overproduction of lactic acid. Metabolically engineered *E. coli* with a new acrylate pathway, that converts the substrate lactate to propionic acid, showed remarkable changes in metabolic fluxes. A notable change in flux distribution around pyruvate node, leading to an increase in lactate production was observed. Now we have to improve the lactate production by bioprocess strategies. The experiment was done in two phases that is aerobic and anaerobic phase. In aerobic phase 20gm/l and anaerobic phase 30gm/l glucose was used. The inoculum was inoculated to bioreactor when culture OD reached at 1.5. Anaerobic phase was created by sparging N₂ after reaching at OD (600nm) 10. After 2 hours of adaptation 0.5mM IPTG (1molar stock) concentration was used for maximum enzymatic activity for lactic acid production. OD was increased slowly but after induction it reached maximum value that is log phase and after that it reached constant. In adaptation phase OD was also constant. A maximum production level of 12g/l in 12hrs batch fermentation was obtained which is 66% higher than the control.

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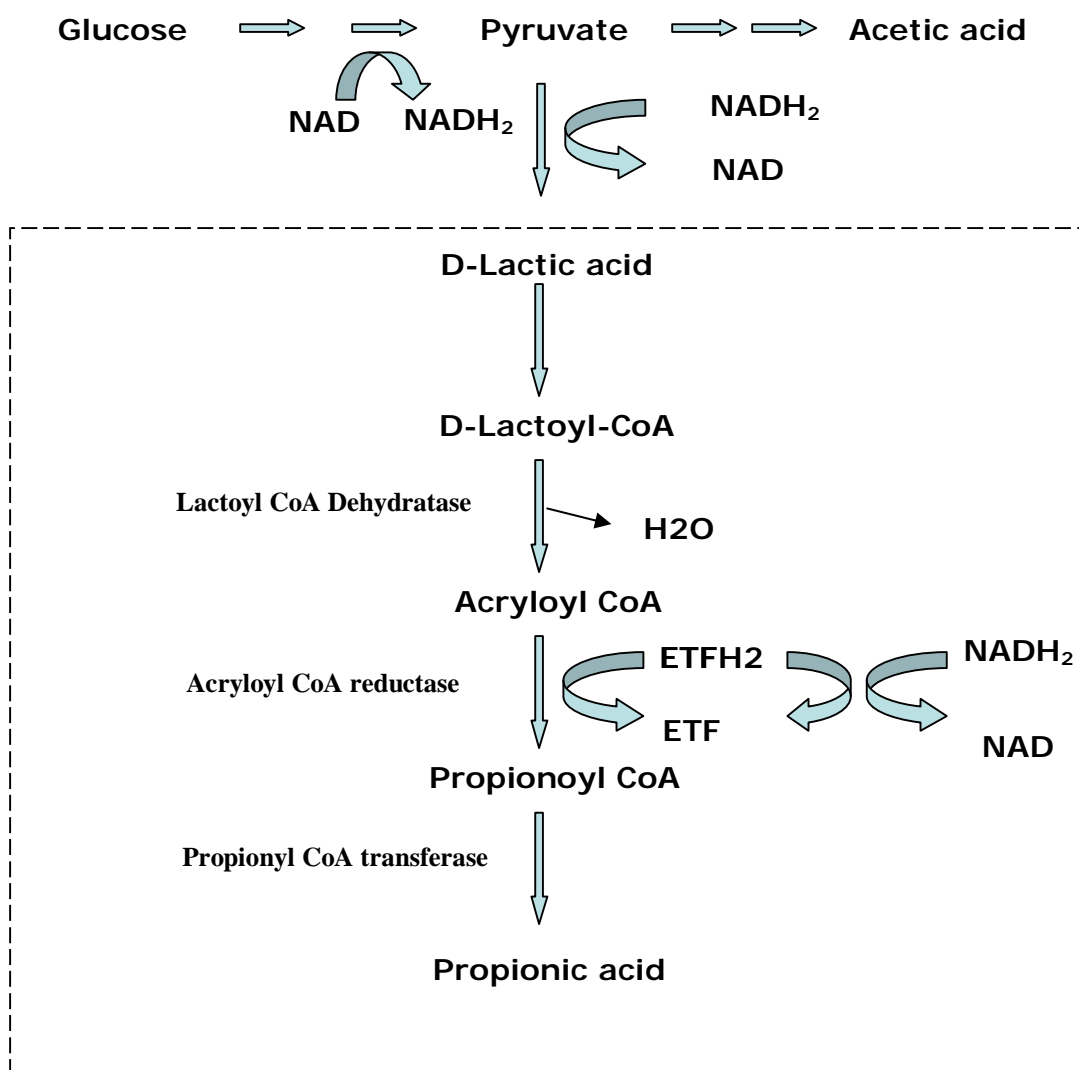


Fig. 1: Acrylate Pathway

Screw cap vial studies: Genes encoding the different enzymes of acrylate pathway were analysed for their specific lactate productivity to find out the enzymes responsible for lactate over production. Following results were reported (Table 1).

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Table1: Genes encoding the different enzyme for their specific lactate productivity

Genes encoding different enzymes combination of Acrylate pathway	Specific Lactate productivity($\text{g g}^{-1}\text{h}^{-1}$)
<ul style="list-style-type: none"> ▪ <i>lcd</i> and <i>acr</i> 	<ul style="list-style-type: none"> ▪ Similar to host(0.45)
<ul style="list-style-type: none"> ▪ <i>acr</i> and <i>pct</i> 	<ul style="list-style-type: none"> ▪ 0.94 (This may be due to Pct since Acr alone did not show any affect.)
<ul style="list-style-type: none"> ▪ <i>lcd</i>, <i>lcdacr</i> and <i>vk-23</i> 	<ul style="list-style-type: none"> ▪ 1.20

Above data are mean of three sets of experiments.

From the data obtained it is intricate to analyse the enzyme responsible for lactate over productivity. Therefore it is hypothesised that it may be due to be a cumulative effect of all three enzymes. So further experiments were designed with strain VK-23 having all three enzymes of acrylate pathway to improve lactate production.

Fed batch fermentation analysis: In Fig. 2 shows the comparison of lactate production rate between the clone and control. The final lactic acid concentration of 24.2g/l was reported which was 95% higher than the control. The average specific (0.55g/g of cells), volumetric productivity (1.66g/litre .h) and yield (0.96g/g of glucose) of clone were almost double of control (Fig. 3). From the production level of various metabolites (Fig. 6) it was observed that introduction of new acrylate pathway has a strong inhibitory effect on pyruvate formate lyase (pfl) along with little effect on other enzymes around pyruvate node but the reason for this cause is unknown. No growth was observed in production phase so average biomass 3g/l was taken for all calculation in Fig 2.

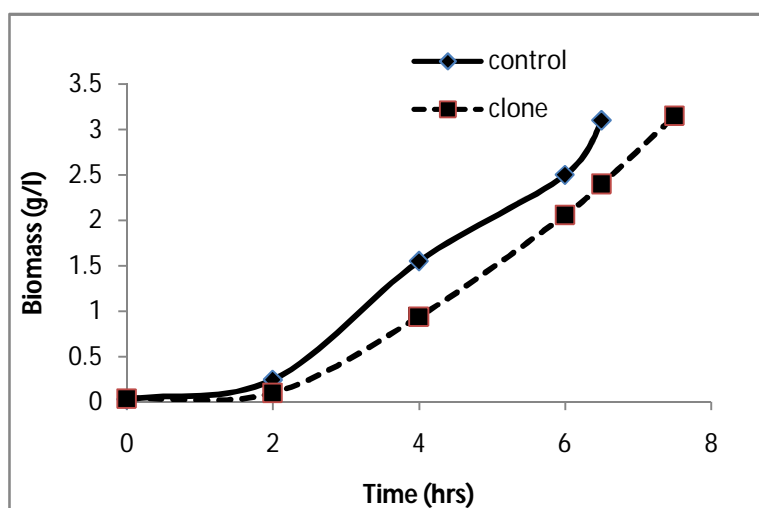


Fig. 2: Growth profile of control and clone during aerobic phase.

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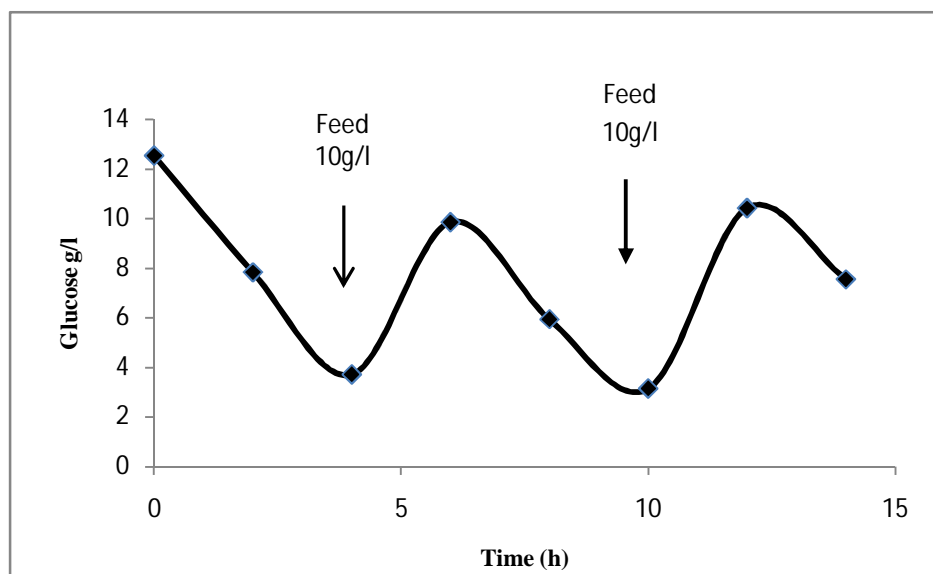


Fig. 3: Glucose levels during production phase

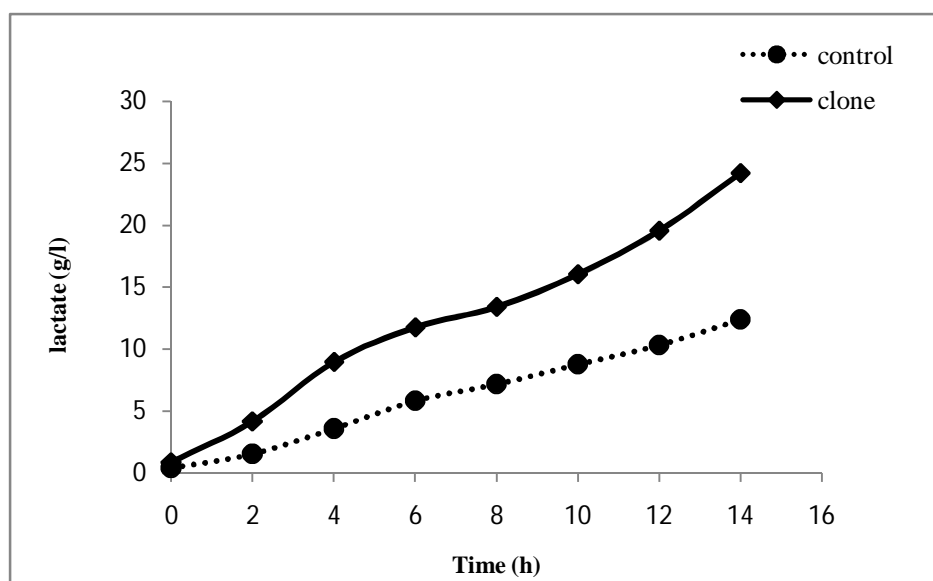


Fig. 4: Lactate production rate of control and clone after induction.

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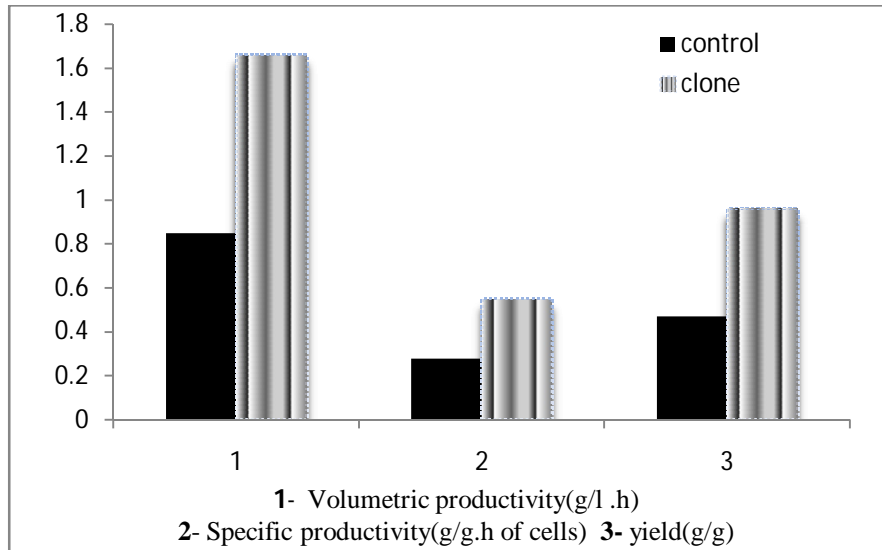


Fig. 5: Average volumetric, specific productivity and yield after induction.

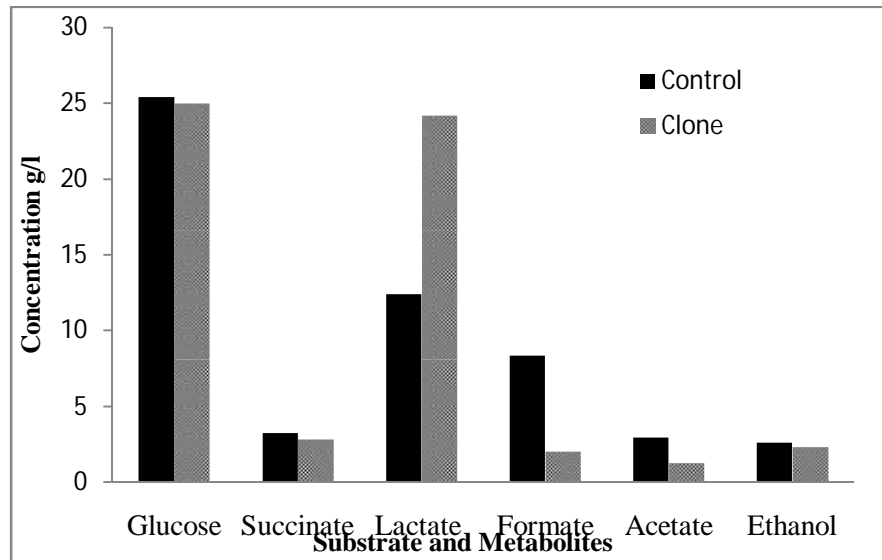


Fig. 6: Comparison of substrate consumption and production levels of various metabolites in fed batch bioreactor studies.

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Data shown in all above graphs are mean of three sets of experiment.

IV. CONCLUSIONS

The current major markets for lactic acid are food related industries, but the emerging markets for poly lactic acid polymer would cause a significant increase in growth of lactic acid consumption. Since only optically pure lactic acid can be polymerised, the primary goal of industries to produce pure isomers, which can be achieved only by biotechnological mode of acid production. Lactic acid bacteria have been used widely for lactic acid production but the requirement of complex media and low growth rate are major constraints. *E.coli* was found to be a suitable host to overcome all. Strain VK-23 with new acrylate pathway has shown great potential to over produce lactic acid. The final lactic acid concentration of 24.2g/l was reported which was 95% higher than the control. The productivity level ($2.4\text{g l}^{-1}\text{h}^{-1}$) obtained in fed batch mode is comparable with metabolically engineered lactic acid overproducers. This can be further improved by using enzyme inhibitors for the competitive pathways at the pyruvate node. So VK-23 has capability to meet the current market demand of fermentation based lactic acid.

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