# Influence of Carbon and Nitrogen Sources on the Growth and Sporulation of *Bacillus thuringiensis* var *Galleriae* for Biopesticide Production

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The influence of glucose and yeast extract (YE) as carbon and nitrogen sources, respectively, on the metabolic processes involving growth, insecticidal protein synthesis and sporulation were studied in batch bioreactor. Significant changes in the metabolic process were observed with different concentration level of glucose and YE. Higher concentration of glucose has resulted in heterogenous population of cells and higher concentration of YE has resulted in the delay of spore release. This detailed study on the influence of the substrate level towards metabolic flux could provide a way for the enhancement of sporulation and insecticidal crystal proteins (ICP). Culture growth as a macroscopic phenomenon was observed to be influenced by varying level of substrates for optimum metabolic flux for the growth and subsequent sporulation with ICP synthesis. Furthermore, need for optimum initial concentration of glucose and YE for the balanced flux of substrates, was realized.

*Keywords:*
*Bacillus thuringiensis* subsp. *galleriae*, biopesticide, insecticidal crystal protein, metabolic flux, sporulation

## Introduction

Metabolic flux identification and analysis has been an excellent tool for the improvement of bioprocess. Recent study on TCA cycle and the glyoxylate shunt was carried out in *E. coli* BL21 and JM109 using either $^{13}C$ – NMR or MS.[1] As $^{13}C$ and NMR studies could not be carried out in actively growing cells,[2] and expensive, qualitative analysis of flux distribution with macroscopic identification of different phases of microbial metabolic activity such as processes like growth, sporulation, product formation and substrate utilization, could be employed. These studies could further reveal the crucial metabolic pathways for the enhancement of product synthesis. Extensive reports indicate the enhancement of microbial productivity by metabolic flux analysis. Metabolic flux distribution was identified to understand the impact of YE and complex organic compounds on lactic acid fermentation[3,4] and the effect of ammonia on cell growth and poly--hydroxybutryic acid production.[5] Influence of glucose and xylose concentration on intracellular fluxes of recombinant *Saccharomyces cerevisiae*[6] and industrial byproducts on the production of polyhydroxyalkanoates,[7] was studied. Flux of glucose favoring high growth was identified with minimum level of intermediates during baker`s yeast production.[8] Studies towards the understanding the impact of C/N ratio with initial concentration of total solids on insecticidal crystal protein (ICPs) and spore production in *Bacillus thuringiensis* with complex medium was reported on intracellular energy basis.[9] Also, existence of nitrogen sources in the medium during the toxin-expression phase was found to be detrimental to the toxin protein expression and its depletion has initiated sporulation and toxin expression with high carbon source encouraged protein expression in genetically engineered *Bt*.[10] Hence, the strategy of understanding the metabolic process on macroscopic scale could extremely influence the process development in biotechnology.

The carbon/energy source and nitrogen sources were necessary for the growth and product formation in microbial cultivation. The nature and characteristics of these substrates has a predominant role to play in the metabolism of microorganism. Particularly, differentiation of the cell into sporulation from vegetative phase, has made its influence complex. The biochemical studies indicated various environmental conditions favoring the initiation of sporulation and subsequent synthesis of insecticidal crystal proteins. The network of biochemical reactions associated with the microbial process, has made the follow-up with reference to the individual components, a difficult one. In addition, internal limiting steps/ bottlenecks could be identified to increase the effectiveness of the biopesticide production process. These limiting steps include expression of enzymes involved in metabolism, intermedi-

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ates/ metabolites etc. This flux of metabolites has a direct reflection on the concentration of substrates. Hence, response of the microbes to substrates could be different, depending upon it`s nature and concentration level.

Previous studies on the nutritional requirements of *Bacillus thuringiensis subsp. galleriae* synthesizing insecticidal crystal protein at the onset of sporulation, has helped in the identification of glucose and YE with amino acids supplementation.[11,12] As amino acids supplementation could not stimulate the metabolic stress encountered by the culture with complex media, YE was provided to serve the needed purpose. This could provide the needed amino acids on hydrolysis and in addition could provide unidentifiable growth supporting factors. Studies were carried out with online monitoring of metabolic heat released during cultivation to facilitate growth phase identification and subsequent enhancement of product formation.[13,14] Even though, significant role of complex media towards higher levels of toxicity has been identified earlier, the seasonal variations and unknown regulatory mechanisms could lead to improper interpretation of the data, obtained on cultivation with complex media. But advancement in analytical techniques, has made possible to simulate the complex medium using chemically defined medium[15] leading to the understanding of the effects of individual components and its interaction. Hence, detailed knowledge on the metabolic process in terms of concentration level of nutrients could be effectively utilized for optimizing the external environment in order to facilitate the favorable condition for enhanced metabolic activity. Hence, bioprocess improvement for enhancing biomass and insecticidial crystal protein (ICP) concentration in terms of the macroscopic environment of substrate concentration, was performed. Experimental design was employed to identify the influence of components incorporated in the medium in comparison to single dimensional approach.

## Materials and methods

### Microorganism and media

*Bacillus thuringiensis* var. *galleriae* was obtained from the laboratory strain collection and maintained on Nutrient Broth (NB) agar plates at 4 °C. The culture was also maintained as spore stock at –20 °C. Nutrient Broth (NB) composition (g $l^{-1}$): Peptone 10; YE 3; NaCl 5 (pH 7.2) Salts and trace elements composition in the medium were taken from Sachidanandham *et al.*[12] This medium was supplemented with varying concentration of glucose and YE in our studies.

### Cultivation conditions and Inoculum

Cultivations were carried out in 1.5 l bioreactor (Bioengineering AG, Wald, Switzerland) with a working volume of 1.0 l. Cultivation was started with 500 rpm as agitation and 1 l $l^{-1}$ $min^{-1}$ as aeration. Dissolved oxygen concentration in the bioreactor was maintained above 30 % of saturation by adjusting aeration and agitation manually. Temperature and pH was maintained at 30 °C and 7.0 respectively during the cultivation. Foam was controlled by the manual addition of $\varphi$ = 10 % polypropylene glycol. 100 ml of exponentially grown culture in NB medium from a single colony of *Btg* maintained in NB agar plate was used as inoculum.

## Analytical methods

Biomass concentration in terms of optical density was measured using spectrophotometer (Hitachi U2000) and correlated with cell dry mass as: Cell density, g $l^{-1}$ = 0.1933 · O.D + 0.0444 [$R$ – squared: 0.9963]. Residual glucose mass concentration was estimated in the biochemistry analyzer (Yellow Springs Instruments Inc.). Spores were counted from the colonies developed in NB agar on incubation from the heat-treated (70 °C for 10 min) samples. The ICP concentration was estimated using enzyme linked immunosorbent assay (ELISA) based quantification method.[16,17] ICP purified, using NaBr density gradient centrifugation,[11,18] was used as the standard in the above estimation. Microscopic observation was carried out periodically using Leitz microscope.

### Batch experiments

Two-dimensional approach was employed for understanding the influence of carbon and nitrogen source on the metabolic process. Carbon source, glucose and nitrogen source, YE, were selected in the range 3.4 – 34 g $l^{-1}$ and 1.0 – 20 g $l^{-1}$, respectively, with salts and trace elements as mentioned earlier. The above concentrations of the substrates were selected to identify its metabolic role on inhibition and limitation. Statistical based full factorial experimental design was developed for different combinations and concentration level of the substrates. According to the design suggested by the statistical software STATGRAPHICS 3.1, batch cultivations were performed in the bioreactor under controlled conditions. Each run was designated as GY1 to GY9 and initial concentration levels of glucose and YE employed in each cultivation are shown in Table 1. Interaction between the carbon and nitrogen sources involved in the medium with respect to estimated macroscopic component was

Table 1 – *The initial concentration levels of glucose and yeast extract as C- source and N – source, respectively, used in batch cultivations. This levels were obtained from the experimental design employed for the concentration range of 3.4 – 34.0 g* $l^{-1}$ *for glucose and 1.0 – 20 g* $l^{-1}$ *for yeast extract.*

| S. No | Medium | Initial Glucose mass concentration $\gamma$ / g $l^{-1}$ | Initial Yeast Extract mass concentration $\gamma$ / g $l^{-1}$ |
|---|---|---|---|
| 1. | GY1 | 3.4 | 1.0 |
| 2. | GY2 | 3.4 | 10.5 |
| 3. | GY3 | 3.4 | 20.0 |
| 4. | GY4 | 18.7 | 20.0 |
| 5. | GY5 | 18.7 | 10.5 |
| 6. | GY6 | 18.7 | 1.0 |
| 7. | GY7 | 34.0 | 1.0 |
| 8. | GY8 | 34.0 | 10.5 |
| 9. | GY9 | 34.0 | 20 |

established using a second order polynomial equation using the above software.

## Results and discussion

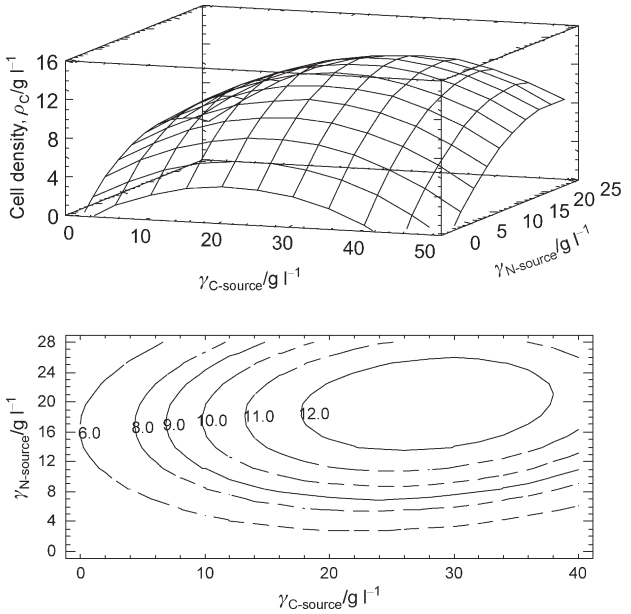
The substrate as either carbon or nitrogen source has to proceed through a network of biochemical reactions to result in a macroscopic change in the microbial environment. In addition, environmental conditions favor this substrate flux in different directions for needed activities during cultivation. Hence, batch cultivations were followed by variations in pH (direction of change) and dissolved oxygen concentration, DO. This was subsequently interpreted in terms of metabolic fluxes of the intermediates/ metabolites during different phases of growth. Even though pH was maintained at 7.0, direction of change in pH could give information, regarding the secretion and subsequent consumption of acidic intermediates. The estimated values on analysis of the samples from the individual cultivations with varying initial concentration levels of glucose and YE, are indicated in Table 2.

### Effect on cell density

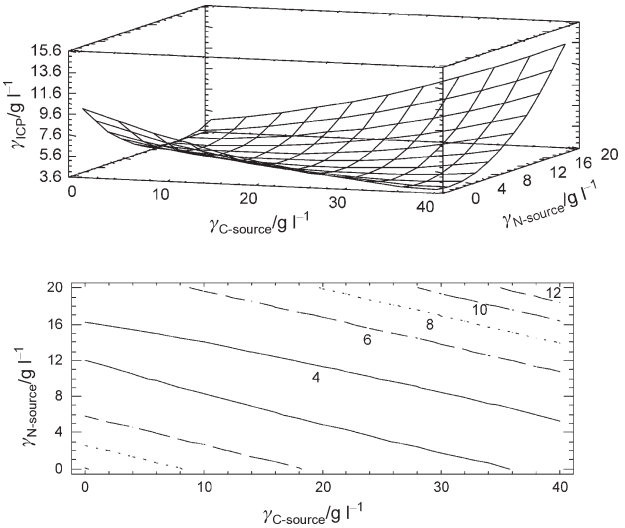
The influence of substrates on maximum cell density obtained in the batch cultivation could be observed from the Figure 1. It was estimated that a maximum cell density of 12.92 g $l^{-1}$ could be obtained with glucose at 27.85 g $l^{-1}$ and YE at 19.7196 g $l^{-1}$. Further, it was observed that increasing the concentration of, both, carbon and nitrogen sources has resulted in its increase and followed by its decrease beyond the maximum point with further increase in carbon and nitrogen sources, due to inhibitory effects. The contour plot indicated the increase in cell density with subsequent increase in either of the above variables due to the consumption of glucose and YE for biomass synthesis substantiated by $Y_{x/s}$ being greater than 1.0 in the respective cases. In particular, rate of increase in maximum cell density achieved was observed to be

Table 2 – *The estimated values on analysis of the samples for the batch cultivations with varying initial concentration of glucose and yeast extract as C – source and N – source, respectively, in bench scale bioreactor*

| S.No | Samples | Cell dry mass $\gamma$/g $l^{-1}$ | ICP concentration $\gamma_{ICP}$/g $l^{-1}$ | Spore concentration $C_{spo}$/$ml^{-1}$ | $Y_{p/s}$ | $Y_{p/x}$ | Specific growth rate $hr^{-1}$ |
|---|---|---|---|---|---|---|---|
| 1. | GY1 | 2.4684 | 9.1328 | 9.067E+08 | 2.7262 | 3.6999 | 0.5676 |
| 2. | GY2 | 5.4568 | 2.5255 | 6.500E+09 | 0.7406 | 0.4628 | 0.8179 |
| 3. | GY3 | 7.3511 | 6.0175 | 1.344E+12 | 1.8070 | 0.8186 | 1.0647 |
| 4. | GY4 | 13.0922 | 6.3290 | 2.513E+12 | 0.3497 | 0.4834 | 0.8198 |
| 5. | GY5 | 10.8885 | 6.7444 | 1.360E+12 | 0.4133 | 0.6194 | 0.7775 |
| 6. | GY6 | 3.0792 | 3.9313 | 8.420E+10 | 0.7474 | 1.2767 | 0.5686 |
| 7. | GY7 | 3.3576 | 4.6383 | 7.296E+11 | 1.0787 | 1.3814 | 0.5635 |
| 8. | GY8 | 10.8885 | 3.5651 | 2.625E+13 | 0.1840 | 0.3274 | 0.8077 |
| 9. | GY9 | 11.6231 | 12.3999 | 1.480E+13 | 0.3943 | 1.0668 | 0.9229 |




F i g . 1 – *The response surface and contour plots for cell density with varying initial concentration of glucose and yeast extract as C – source and N – source, respectively, in batch cultivations, supplemented with salts and trace elements [R-squared=94.94%]. The values inside the contour plot indicate constant cell density.*




F i g . 2 – *The response surface and contour plots for specific growth rate of the culture during the exponential phase with varying initial concentration of glucose and yeast extract as C – source and N – source, respectively, in batch cultivations supplemented with salts and trace elements. The values inside the contour plot indicate constant specific growth rate.*

relatively high at an optimum ratio of carbon and nitrogen sources. This indicated the balanced role of carbon and nitrogen sources in the flux of metabolic resources towards biomass synthesis. In particular, heterogenity in cell population was observed during sporulation phase with batch cultivations, carried out at high initial concentrations of glucose and YE and this could be due to the impact of enhanced metabolic stress. In particular, prominent influence of carbon source was observed in comparison with the nitrogen source at higher concentration levels due to its inhibition. Higher initial concentration of, both, the glucose and YE has resulted in lag phase during the batch cultivation. But, higher initial concentration of YE at 20 and 10.5 g l$^{-1}$ has resulted in two distinct growth phases. Also, analysis of biomass productivity in the same ground as that of achieved cell density indicated the similar relationship (data not shown).

## Effect on specific growth rate

The dependency of rate of biomass formation, as specific growth rate of the culture during logarithmic phase on the nutrient sources, was studied by correlating the estimated specific growth rate in terms of initial concentration levels of carbon and nitrogen sources. The dependency of specific growth rate on the carbon and nitrogen sources was found to be complex due to the saddle (min-max) point in the Figure 2. The influence of nitrogen source on specific growth rate of the culture was observed to be significant in comparison with the carbon source. With nitrogen source at high concentration, relatively high specific growth rates were noticed with high and low levels of glucose. These high specific growth rates could be due to the consumption of YE as carbon source for biomass synthesis as reported by *Mignone & Avignone*.[19] As ICP was synthesized at the onset of sporulation from the vegetative phase on differentiation, condition prevailing during this transition period has a predominant role in the spore formation. Significantly, increase in specific growth rate was observed with increase in YE conc. at 3.4 and 34 g l$^{-1}$ conc. of glucose. But at 18.7 g l$^{-1}$ glucose concentration increase in growth rate was found to be relatively less. As a significant observation, high specific growth rate has not contributed towards ICP synthesis supporting previously discussed result. Hence, impact of carbon and nitrogen source on specific growth rate was observed to be crucial with redirection in metabolic fluxes at varying concentrations of glucose and YE.

## Effect on ICP concentration

The non-growth associated synthesis of ICP during sporulation has made its correlation with cell density a difficult task. Correlation of ICP concentration with initial concentration of glucose and YE indicated a point of minimum with 3.647 g l$^{-1}$ at 31.234 g l$^{-1}$ of glucose and 4.7221 g l$^{-1}$ of YE. The ICP concentration profile (Figure 3) was observed to be in contrast to the cell density profile, thereby indicating the complex relationship between bio-

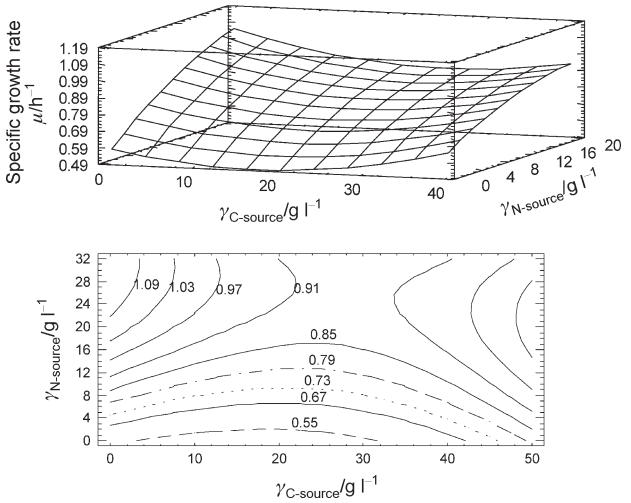



Fig. 3 – *The response surface and contour plots for insecticidal crystal protein (ICP) concentration with varying initial concentration of glucose and yeast extract as C – source and N – source, respectively, in batch cultivations supplemented with salts and trace elements [R-squared=74.19 %]. The values inside the contour plot indicate constant ICP concentrations.*

mass and ICP synthesis. ICP concentration was found to be increasing towards the lower and higher concentration of substrates from the point of minimum. In addition, increase in nitrogen source was more pronounced in ICP concentration in comparison with increase in carbon source. It was reported that 80 % of amino acids needed for ICP synthesis has been obtained from the vegetative protein turnover during sporulation,[20] indicating minimum external influence of nitrogen source in terms of amino acids and short peptides on the synthesis of ICP. Hence, process of ICP synthesis could have been influenced by the high concentration of nitrogen source, rather, than its contribution in the form of amino acids and short peptides. In addition, nitrogen source could have influenced the synthesis of spore components leading to delayed spore release due to its high concentration. Point of minimum ICP concentration was observed to occur near the point of maximum cell density with respect to initial glucose and YE concentration. This indicates that the attainment of high cell density need not necessarily result in enhanced synthesis of ICP. The enhanced metabolic stress imposed by the variations in metabolic flux of substrates, could have resulted in the loss of mega-plasmid, thereby, segregating into variants with decreased concentration of ICP.

## Effect on $Y_{p/x}$

Analysis of $Y_{p/x}$ (data not shown) also revealed that the metabolic flux favoring biomass hasn`t contributed for ICP synthesis with minimum $Y_{p/x}$ at the carbon and nitrogen concentration favoring high cell density. Increase in carbon and nitrogen sources has resulted in less $Y_{p/x}$, thereby, favoring biomass synthesis till the point of minimum. Hence, metabolic flux on minimum point could indicate its redirection towards biomass and preventing the initiation of sporulation. Further from the point of minimum, $Y_{p/x}$ increased with relatively less cell density at higher concentrations of carbon and nitrogen sources. This could be due to the enhanced flux of nutrients directed towards ICP synthesis. In particular, with lower concentration of glucose increase in YE has resulted in significant decrease in $Y_{p/x}$, thereby, indicating that the culture under the limitation of carbon source has taken the nitrogen source for its growth. Also, rate of utilization of glucose was observed to increase with increase in YE concentration, thereby, making the role of YE in metabolic process of, both, biomass and ICP as a significant one.

## Effect on spore concentration

The involvement of differentiation process on spore formation and ICP synthesis has necessitated the understanding of the influence of initial concentration levels of carbon and nitrogen sources on yield of spores (Figure 4). Relatively high spore count was observed with high initial concentration of carbon source due to accumulation of acidic intermediates during growth phase, thereby, influencing the sporulation associated events. In particular, increase in nitrogen source has relatively insignifi-

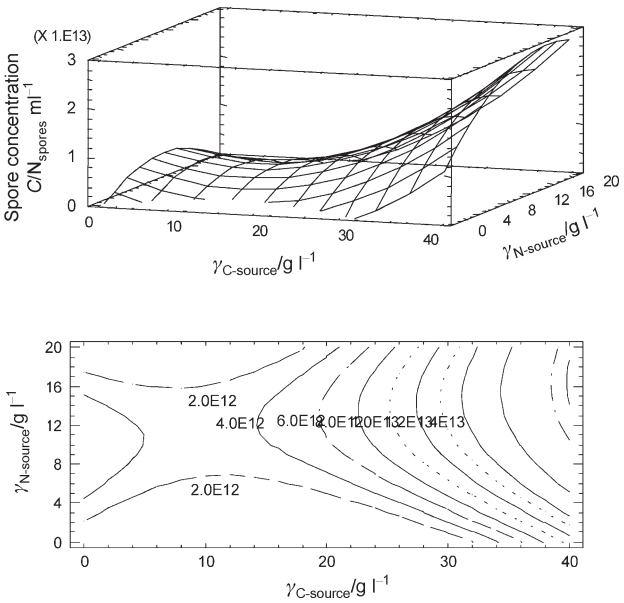



Fig. 4 – *The response surface and contour plots for concentration of spores with varying initial concentration of glucose and yeast extract as C – source and N – source, respectively, in batch cultivations supplemented with salts and trace elements. The values inside the contour plot indicate constant spore concentration.*

cant influence on the yield of spores. High initial concentration of glucose has resulted in the heterogenous population of vegetative, sporulating cells and spores during the later phase of the cultivation. In contrast, high initial concentration of YE has made the process of spore release as difficult due to impaired synthesis of spore components. In some cases, high spore count has not resulted in high concentration of ICP and this could be due to the impaired synthesis of ICP. It was also observed in batch cultivation that there was significant glucose left unconsumed, even after sporulation in the medium with less initial concentration of YE, indicating the need for balanced flux of nutrients.

### Effect on $Y_{p/s}$

With interdependent activities happening during the cultivation, flux of glucose towards ICP was studied by the product yield coefficient ($Y_{p/s}$) from the Figure 5. It was observed that increasing the initial concentration of carbon and nitrogen source has resulted in decrease of $Y_{p/s}$. $Y_{p/s}$ was observed to be less at high mass concentration of YE at 20 g $l^{-1}$ with glucose concentration at 18.7 and 34 g $l^{-1}$ and sufficiently high at low concentration of 3.4 g $l^{-1}$ glucose. In addition, rate of decrease in $Y_{p/s}$ was observed to be relatively high at a particular ratio of carbon to nitrogen source. Further, $Y_{p/s}$ was observed to be minimum near the concentration level of carbon and nitrogen sources needed for obtaining maximum cell density. This clearly demonstrated the flux of metabolic resources towards biomass away from the synthesis of ICP substantiating the results mentioned earlier.

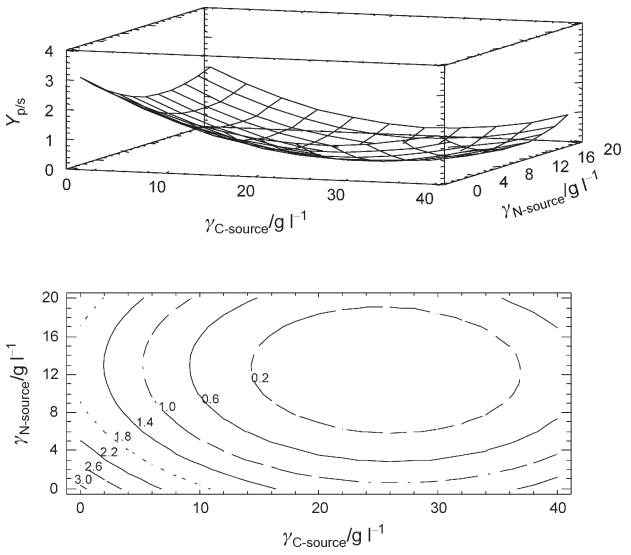



Fig. 5 – *The response surface and contour plots for product yield coefficient ($Y_{p/s}$) with varying initial concentration of glucose and yeast extract as C – source and N – source, respectively, in batch cultivations supplemented with salts and trace elements. The values inside the contour plot indicate constant product yield coefficient.*

During this study, analysis of product kinetics in terms of ICP productivity and spore productivity could not be carried out in relation with initial concentrations of carbon and nitrogen sources. As ICP was synthesized at the onset of sporulation, time of completion of ICP synthesis should be known. Hence, ICP productivity in terms of the cultivation period till harvest could yield erroneous values. Also, delay and improper spore release was observed in cultivations with high concentrations of YE. This has led to the estimate of exact time of spore release difficult. The role of ammonium sulphate and its concentration level on the metabolic process was not studied but maintained at a constant level. Furthermore, data could not be interpreted in terms of the concentration ratio of carbon and nitrogen source, because of the higher influence of its concentration level in comparison with the concentration ratio. The results indicated the significant influence of substrate flux on the macroscopic metabolic processes, and balanced flux between catabolic and anabolic pathways were observed to be necessary for the enhanced metabolic activity. Hence, fedbatch cultivation could be performed in the future to identify and execute the balanced flux for the enhanced synthesis of ICP.

## Conclusions

Studies pertaining to metabolic fluxes in terms of macroscopic processes like growth, ICP synthesis and sporulation, has provided an insight into the metabolic processes involved. The metabolic flux of substrate was found to vary in response to different environmental stimuli. The understanding of metabolic flux with response to different concentration levels of glucose and YE indicated its prominent role on ICP synthesis. Furthermore, the need of constant ratio of the carbon and nitrogen sources was observed for the metabolic flux redirection towards enhanced ICP synthesis. Also, influence of varying level of substrates has pronounced effect on the specific growth rate of the culture. This was due to the changes in the metabolic network during growth and further differentiation into sporulation with ICP synthesis. There was an optimum specific growth rate for providing a balanced metabolic flux towards efficient synthesis of ICP, sporulation and subsequent release of spores. The needed condition interms of initial concentration levels of glucose and YE for the enhanced production of microbial products could be understood. The optimum glucose concentration level providing the balanced substrate flux could be decided on analysis of the

instantaneous growth rate and substrate utilization rate during the batch cultivation. This understanding could lead to the employment of appropriate feeding strategies in fed-batch cultivation towards the enhancement of biomass and ICP synthesis.

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## List of symbols

$y$ – yield
$\gamma$ – mass concentration, g $l^{-1}$
$\varphi$ – volume fraction, %
$C$ – spores concentration, $N_s/V$, $ml^{-1}$
$N_s$ – number spores
$V$ – volume

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