POLYPHASIC CHARACTERIZATION AND OPTIMIZATION OF GROWTH CONDITIONS FOR HIGH CELL DENSITY BATCH CULTIVATION OF *Rhizobium leguminosarum* USING RESPONSE SURFACE METHODOLOGY

Project Report Prepared

at

Institute of Himalayan Bioresource Technology
Palampur (H.P.)

Submitted to

GURU NANAK DEV UNIVERSITY

(AMRITSAR)

in

Partial Fulfillment of the Requirement for the Degree

Of

Master of Science

(FERMENTATION AND MICROBIAL TECHNOLOGY)

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INTRODUCTION

Microorganisms play an important role in providing nutrients to the plants. Consequently, soil fertility largely depends upon its microbial population and their activity. Symbiosis between leguminous plants and rhizobia is of considerable agricultural importance. These nitrogen-fixing soil bacteria, which directly or indirectly stimulate the plant growth, have been targeted as the potential plant growth promoting bacteria (PGPB) (Sahgal *et al*., 2003). Rhizobia were the first microbial inoculants identified and used commercially for improving productivity in legumes (Kannaiyan, 2002). The beneficial effect of rhizobia on legumes in terms of biological nitrogen-fixation is well known (Werner, 1992). *Rhizobium leguminosarum* promotes the growth of various leguminous plants like pea, canola, chickpea, lettuce, and lupine (Hoflich *et al*., 1994; Noel *et al*., 1996). The strains of *Rhizobium* sp. reportedly have the potential to be used as PGPR with non-legumes like corn, rape, wheat, radish, maize, barley and rice (Wiehe and Hoflich, 1995; Yanni *et al*., 1997). Rhizobial strains which produce siderophore and solubilize inorganic phosphate are further useful in plant growth promotion (Jadhav *et al*., 1994; Chabot *et al*., 1996a).

Utilization of microbial-resource in sustainable agriculture requires an assessment of the most suitable conditions for the effective and successful establishment of the PGPR as inoculants in the rhizosphere of the host plant (Tilak *et al*., 2005). Identification and characterization of beneficial microorganisms is important to developing deployment strategies for increasing their contribution in agriculture. Characterization should be based on a more pragmatic approach of deploying a number of methods including phenotypic, chemotaxonomic and genotypic methods for the complete characterization

of microorganisms. Polyphasic approach is the most popular choice for classifying and identifying bacteria and several microbes. Although PGPB may reveal huge potential for crop production, for a microbial inoculant to be commercially feasible, it must be economically mass-produced and formulated into a cost-effective, uniform and readily applicable form. The first step in any such process is to design a suitable medium with optimum growth conditions. Fermentation in research and industry is aimed at maximizing the volumetric productivity that is to obtain the highest possible amount of product in a given volume within a certain time. High cell density culture (HCDC) is required in order to improve the microbial biomass and product formation substantially. The present project was undertaken with the objective of characterizing a plant growth promoting *Rhizobium* strain using polyphasic approach and designing an appropriate growth medium for its mass production by employing the statistical design Response Surface Methodology.

Response Surface Methodology is a combination of statistical and mathematical techniques useful for optimization of bioprocesses and it can be used to evaluate the effect of several factors that influence the responses by varying them simultaneously in limited number of experiments. This methodology has been utilized successfully to optimize composition of microbiological media (He *et al*., 2004), improve fermentation processes (Liong and Shah, 2005) and for developing new products (Rodriguez *et al*., 2006). RSM includes factorial designs and regression analysis which helps in evaluating the effective factors and in building models to study the interactions and select optimum conditions of variables for a desirable response. In the present study a plant growth promoting nitrogen fixing Rhizobium strain was characterized based on polyphasic approach, selection of most significant medium

ingredients affecting growth by one variable at a time, and optimization of culture growth conditions by a full factorial Box-Benhken design of the RSM.

The main components of the work include:

- Morphological, physiological, biochemical and molecular characterization (16S rRNA gene) of the bacterial strain;
- Selection of most significant medium ingredients affecting growth by selecting one variable at a time .
- Optimization of culture growth conditions for high cell density culture under shake flasks;
- Up-scaling in high density cell cultures of the PGPR strain.

REVIEW OF LITERATURE

Rhizobia occur as free-living heterotrophs in the soil but they do not fix atmospheric nitrogen in this state. Under appropriate conditions, however, rhizobia can invade root hairs, initiate the formation of a nodule, and develop nitrogen-fixing activity. The association between rhizobia and plant roots is specific, with mutual recognition between the two compatible partners based on chemotactic response and specific binding to the root hair prior to invasion and establishment of the root nodule. The legume plant root recognizes the right population of rhizobia, which in turn recognizes the right kind of leguminous root. The literature pertinent to the present investigations is reviewed under the following heads:

Polyphasic Characterization

Classification of microorganisms on the basis of traditional microbiological methods creates a blurred image about their taxonomic status and thus needs further clarification. It should be based on a more pragmatic approach of deploying a number of methods for the complete characterization of microbes. Hence, the methods now employed for bacterial systematics include, the complete 16S rRNA gene sequencing and its comparative analysis by phylogenetic trees, DNA-DNA hybridization studies with related organisms, analyses of molecular markers and signature patterns, biochemical assays, physiological and morphological tests. Collectively these genotypic, chemotaxonomic and phenotypic methods for determining taxonomic position of microbes constitute what is known as the 'polyphasic approach' for bacterial systematics. The term "polyphasic taxonomy" was coined by Colwell (1970) and is used for delineation of taxa at all levels (Murray *et al*., 1990). The first attempt of

microbial classification based on single-stranded DNA was made by Schildkraut and coworkers in 1961. This was a major breakthrough in the world of microbial classification paving the way towards development of the polyphasic system of classification in its present form. This approach is currently the most popular choice for classifying bacteria and several microbes, which were previously placed under invalid taxa have now been resolved into new genera and species. This has been possible owing to rapid development in molecular biological techniques, automation of DNA sequencing coupled with advances in bioinformatic tools and access to sequence databases. Several DNA-based typing methods are known; these provide information for delineating bacteria into different genera and species and have the potential to resolve differences among the strains of a species. Therefore, newly isolated strains must be classified on the basis of the polyphasic approach. A number of criteria that include phenotypic, chemotypic and genotypic features used for polyphasic characterization of bacteria are discussed below:

Phenotypic methods

Phenotype is the observable expression of genotype and it includes morphological, physiological and biochemical properties of the organism. Before the advancement in molecular techniques, bacterial taxonomy was solely based on comparative studies of the phenotypic features and this practice was directly linked to laboratory pure cultures. Therefore, it was biased towards aerobic heterotrophic microorganisms (Vandamme *et al.,* 1996; Mora and Amann, 2001). Traditional phenotypic tests used in classical microbiological laboratories for this kind of analysis include characteristics of the organism on different growth substrates, growth range of microorganisms on different conditions of salt, pH and temperature and susceptibility towards different kind of

antimicrobial agents etc. One of the major disadvantages with phenotypic methods is the conditional nature of gene expression wherein the same organism might show different phenotypic characters in different environmental conditions. One must note that phenotypic data must be compared with similar set of data from type strain of closely related organism(s). Reproducibility of results between different laboratories is another problem, therefore, only standardized procedure should be used during execution of experiment (Bishop *et al.*, 1962; Collins *et al.*, 1981). Elsevier (2004) characterized a novel plant growth promoting bacteria strain *Delftia tsuruhatensis* HR4 which was isolated from the rhizoplane of rice in north China. Park *et al.* (2004) isolated and characterized growth promoting bacteria on the basis of colony morphology, motility and carbon source utilization pattern from rhizosphere of agricultural crops of Korea.

Chemotaxonomy

The term chemotaxonomy refers to application of analytical methods for collecting information on different chemical constituents or chemotaxonomic markers of bacterial cells in order to group or organize them into different taxonomic ranks (Vandamme *et al.,* 1996; Mora and Amann, 2001). The principle of chemotaxonomy is based on uneven distribution of these markers among different microbial groups (Goodfellow *et al.*, 1993). The use of these analytical methods varies from group to group. The most commonly used chemical markers include cell wall/membrane components such as peptidoglycan (Schleifer *et al.*, 1972) teichoic acids, polar lipids, composition and relative ratios of fatty acids, lipopolysaccharide, isoprenoid quinones and polyamines (Suzuki *et al.*, 1993; Busse and Auling, 1988). Teichoic acids are the polymers of glycerol and are specifically used for characterizing Gram-positive bacteria. Respiratory

quinones which belong to a class of terpenoid lipids are constituents of bacterial plasma membrane and are valuable in microbial systematics (Bishop *et al.*, 1962). For instance, all sulphur-containing bacteria are characterized by the presence of caldariellaquinone, an unusual terpenoid. Analysis of these compounds by using different chromatographic techniques can successfully delineate the bacteria upto the rank of genus.

Whole cell fatty acid analysis is increasingly used both in taxonomic studies and identification analysis (Welch, 1991). The procedures for extraction, chromatographic separation and data analysis are relatively simple and fairly inexpensive screening method. This method allows comparison and clustering of large number of strains with minimal effort and yields descriptive information to characterize the organism. For some genera whole cell fatty acid analysis allows differentiation and identification of individual species or even subspecies and for some organisms, different species have identical fatty acid profiles (Welch, 1991).Importantly, strains must be grown under highly standardized conditions to obtain reproducible results. The commercial microbial identification system gives strict guidelines and provides databases containing species specific fatty acid patterns. Elsevier (2004) analyzed cellular fatty acid composition of bacterial isolate strain *Delftia tsuruhatensis* HR4 using Sherlock system. Park *et al.* (2004) characterized growth promoting bacteria from rhizosphere of agricultural crops of Korea using FAME analysis. Strains were inoculated onto trypticase soya agar and grown for 48 hours for FAME analysis.

Genotypic methods

Several definitions for bacterial species were proposed earlier but with the advent of genotypic methods like 16SrRNA gene sequencing and DNA-DNA hybridization, the concept of bacterial species has been refined. A ‘bacterial species’ is defined as a group of

strains sharing 70% or more DNA-DNA relatedness with 5 °C or less ÄTm value (Tm is the melting temperature of the hybrid) among members of the group, provided that all the phenotypic and chemotaxonomic features agree with the above definition. This is further corroborated from data on 16S rRNA gene sequence analysis wherein bacterial strains showing more than 3% sequence divergence are considered to be the members of different species (Stackebrandt, 1994; Gevers *et al*., 2005; Coenye *et al*., 2005)

16S rRNA sequencing

In 1980s, studies concluded that phylogenetic comparison based on conserved part of genome was much stable than classification solely based on phenotypic traits and other features (Dubnau *et al.*, 1965; Woese, 1987; Clarridge, 2004) Hence the use of rRNA molecules was propagated for making phylogenetic comparisons (Woese, 1987). All the three kinds of rRNA molecules i.e., 5S, 16S, 23S and spacers between these can be used for phylogenetic analyses but the small and large size of 5S rRNA (120 bp) and 23S rRNA (3300 bp) respectively have restricted their use. 16S rRNA gene (1650bp) is the most commonly used marker that has revolutionized the field of microbial systematic (Woese, 1985; Amann *et al.*; 1995 Mora and Amann, 2001). Dubnau and coworkers in 1965 for the first time reported the conservation in the 16S rRNA gene sequence in *Bacillus* (Dubnau *et al.*, 1965) Its widespread use in bacterial taxonomy started after the pioneering work of Woese in 1987 (Woese *et al.*, 1985). 16S rRNA gene sequence is thus used characterizing and identifying bacteria based on its universal distribution, highly conserved nature, fundamental role of ribosome in protein synthesis, no horizontal transfer and its rate of evolution which represents an appropriate level of variation between organisms (Stackebrandt, 1994; Mora and Amann, 2001; Clarridge, 2004). The 16S rRNA molecule comprises of variable and conserved regions and

universal primers for amplification of full 16S rRNA gene are usually chosen from conserved region while the variable region is used for comparative taxonomy. The 16S rRNA gene sequence is deposited in databases such as Ribosomal Database Project II (http://rdp.cme.msu.edu/) and GenBank (http://www.ncbi.nlm.nih.gov/). Sequences of related species for comparative phylogenetic analysis can also be retrieved from these databases.

High Cell Density Culture (HCDC)

Although PGPR may reveal huge potential for crop production for microbial inoculants to be commercially feasible, it must be economically mass-produced and formulated into a cost-effective, uniform and readily applicable form. Productivity is a function of cell density and specific productivity so increasing cell density increases productivity. High cell density culture (HCDC) was first established for yeasts to produce SCP, ethanol and biomass. Later, dense cultures of other mesophiles producing various types of products were developed. Dense cultures of methylotrophs led to the efficient production of polyhydroxyalkanoic acids (Lee and Chang, 1995). Although most HCDC are associated with *E. coli* other microorganisms have the ability to be grown to high cell densities. Bacteria such as *Bacillus subtilis*, *Pseudomonas putida*, α-plantarum, yeasts such as *Saccharomyces cerevisiae*, *Pischia pastoris* and other eukaryotic cells have been reported which can grow to a high cell density. However, there are also a lot of microorganisms that can only be grown to concentrations of the order of few grams per liter although considerable effort has gone into the development of specific fermentation strategies. Several bacteria, archea and yeasts were cultivated to very high cell densities, that is, dry biomass concentrations higher than 100 g/l .e.g. mesophiles like *E. coli* were cultivated on glucose/mineral salt medium in a DO-stat fed batch using dialysis reactor. The maximum CDW obtained was 190 (g/l) in 30 hours with

biomass productivity of 25.2 g/l/day. The maximum biomass concentration does not always correlate with the maximum productivity, as is the case for microorganisms that grow very slowly like archea for fed batch cultures combined with dialysis for continuous cultures because of the higher media volumes needed. The main problems arising from HCDC are solubility of solid and gaseous substrates in watery media, limitation of substrates with respect to growth, instability and volatily of substrates and products, accumulation of products or metabolic by product to a growth-inhibitory level, degradation of products, high evolution rates of CO_2 and heat, high O_2 demand as well as the increasing viscosity of medium in very dense cultures. The fermentation medium determines the chemical or nutritional environment and is thus vital to research into and the manufacturing of microbial metabolites. Chemically defined medium simplify feeding strategies because they are associated with known yield coefficients, growth rates etc. Since high amounts of substrates are needed to reach a high concentration of biomass, these substrates must be fed in a controlled manner after consumption of initial amount in the basal medium. Restrepo *et al.* (2005) reported a 3-step culture protocol consistently producing high cell density culture of *Penicillin* resistant *Streptococcus pneumoniae*. Simone *et al*. (2004) studied high cell density cultures of *Pseudomonas putida* IPT 046 for the production of medium chain length Polyhydroxyalkanoates (PHA's) using an equimolar mixture of glucose and fructose as carbon sources. Kinetic studies of *Pseudomonas putida* growth resulted in a maximum specific growth rate of 0.65/h. Limitation and inhibition owing to NH_4^+ ions were observed, respectively, at 400 and 350 mg of NH_4^+/l. The main concentration of DO in the broth must be 15% of saturation.

Response Surface Methodology

Different strategies can be used for the optimization of cultivation conditions. Conventional "one variable at a time" approach has been traditionally used (Gokhale *et al*. 1991; Pham *et* al., 1998). Use of one variable at a time approach in combination with use of statistical methods, enables easy selection of important parameters from a large number of factors and also the interactions between important variables can be understood. A number of statistical experimental designs have been used for optimizing fermentation variables (Pham *et al*., 1998; Xu *et al*., 2002; Rao *et al*., 2004). Response Surface Methodology (RSM) is a combination of statistical and mathematical techniques useful for optimization of bioprocesses and it can be used to evaluate the effect of several factors that influence the responses by varying them simultaneously in limited number of experiments. This methodology has been utilized successfully to optimize composition of microbiological media (He *et al*., 2004), improve fermentation processes (Liong and Shah, 2005) and for developing new products (Rodriguez *et al*., 2006). Chadha *et al*., (2006) reported the optimization of culture conditions for the production of cellulases and xylanases by *Scytalidium thermophilum* isolated from composted soil, using RSM. Initial experiments showed that culture medium containing rice straw and wheat bran (1:3) as carbon source prepared in a synthetic basal medium supported maximal enzyme production at 45 ºC. Further optimization of enzyme production was carried out using Box–Behnken design of experiments to study the influence of process variables (inoculum level, $(NH_4)_2SO_4$ and pH) on enzyme production. The RSM plots revealed the conditions for obtaining optimum enzyme levels. The models computed for R^2 values ranged between 95% and 98.7% indicating that they are appropriate and can be useful to predict the effect of inoculum level, $(NH_4)_2SO_4$ and pH on enzyme production . Duta *et al.* (2006) while studying the combined

effect of the process parameters for exopolysaccharides production by *Rhizobium* sp. using Response Surface Methodology foung that maximum response for exopolysaccharides production was obtained when highest values of calcium carbonate concentration (1.1g/l), aeration (1.3vvm) and agitation (800 rpm) were applied.

RSM includes factorial designs and regression analysis which helps in evaluating the effective factors and in building models to study the interactions and select optimum conditions of variables for a desirable response. The principle of RSM was described by Khuri and Cornell (1995). An empirical second order polynomial model for three factors was in following form:

$y_i = a_o + \sum_{i=1} a_i x_i + \sum_{i=1} \sum_{j=1} a_{ij} x_i x_j$

where y_i was the predicted response used as a dependent variable; x_i(i=1,2 & 3) and a_{ij} (i=1,2,3;j=i.3) were the model coefficient parameters. The coefficient parameters were estimated by multiple linear regression analysis using the least squares method. Preetha *et al*. (2006) reported optimization of C and N sources and growth factors for enhanced production of an aquaculture probiotic (*Pseudomonas* MCCB 103) using Response Surface Methodology.

Designs of Response Surface Methodology

Box–Behnken Design and Central composite Design (CCD) are efficient designs for fitting second order polynomials to response surfaces, because they use relatively small number of observations to estimate the parameters In many experimental situations, the experimenter is often trying to discover the polynomial relationship between a dependent or predictor variables,x_1,x_2……, x_k. A 2^k factorial design or a fraction of such, can be used to detect which predictor variables, or which two variable interactions have an effect on the response. Elibol *et al*. (2004) reported the optimization of medium composition for the production of

actinohordin by *Streptomyces coelicolor*. A 2^4 full factorial CCD was chosen to explain the combined effects of four medium constituents, viz. sucrose, glucose, and yeast extract and peptone and to design a minimum number of experiments. The optimized medium consisting of 339 g/l sucrose, 1.0 g/l glucose, 1.95 g/l yeast extract and 2.72 g/l peptone predicted 195 ml/l of actinohordin which was 32% higher than that of unoptimised medium. These designs are efficient in that they use few experimental observations, but they cannot detect or estimate any quadratic relationships. After the initial exploration of predictor variables, a CCD or a Box–Behnken Design can be used to investigate the full second order polynomial relationship between the response and the predictor variables. For K=2 we have

$$G = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2$$

Kumar and Satyanarayana (2007) studied the optimization of culture variables for improving glucoamylase production by alginate entrapped *Thermomucor indicae – seudaticae*. The critical variables that affected glucoamylase were identified by Plackett-Burman design (Sucrose, yeast extract, K_2HPO_4 and asparagines) and further optimized by using a four factor CCD of RSM. Immobilized sporangiospores secreted 41% and 60% higher glucoamylase titers in shake flasks and air lift fermenter, respectively, when the variables were used at their optimum. Glucoamylase production (26.3 U/ml) in the optimized medium was in good agreement with the values predicted by the quadratic model, thereby confirming its validity. Protease production by *Aspergillus oryzae* was optimized in shake flask cultures using Box–Behnken experimental design of RSM by Babu *et al.* (2006).

The central composite design of RSM was employed to maximize the biomass production and antagonistic activity. Both biomass and antagonistic compound production of *Pseudomonas* MCCB 103 were maximum at low concentration of NaCl (5g/l). The newly

designed media using RSM provided 19% increase in its biomass and five fold increase in antagonistic compound production (Preetha *et al*., 2006).

Up scaling

The scale up of microbial inoculants is carried out at fermenter level by varying different parameters in order to reach high cell density. Xiao *et al.* (2005) reported optimization of medium constituents for a novel lipopeptide production of *Bacillus subtilis* MO-01 using RSM. A five level four factor central composite design was employed to determine maximum lipopeptide yield. Lee et al (2000) applied phosphorous limitation during fed batch culture by reducing initial KH_2PO_4 concentration in order to increase the polyhydroxyalkanoates concentration by increasing cell density. Konstantinov *et al*. (1990) introduced the balanced DO- stat method which guaranties oxygen supply and prevents overfeeding. They measured the exit gas composition from the fermenter in real time, estimated the glucose uptake rate and determined nutrient feed rate. Guo–Ying *et al*. (2008) reported process optimization for production of L-cysteine by -*Pseudomonas* sp. Zjwp -14 using Response Surface Methodology. The maximum intracellular enzyme activity of 903.6 U/ml in experiment was obtained under optimal conditions with an increase of 15.6% as compared to the original medium components. Cultured *Pseudomonas* species Zjwp-14 cells were applied for biotransformation of DL-ATC to L-cysteine in 5-l fermenter. The molar yield of L-cysteine reached 57.4% with DL-ATC concentration of 10g/l for 6 hours. The authors also observed that cultivation time of *Pseudomonas* sp. Zjwp -14 with maximum enzyme activity was shorter for 6 hrs than in Erlenmeyer flasks. Abadias *et al*. (2003) reported the optimization of growth conditions of the post harvest biocontrol agent *Candida sake* CPA-1 in a lab scale fermenter. Growth conditions (aeration, agitation speed and inoculum size) were studied in batch conditions in a 5 l fermenter using molasses and urea as

growth medium. Consumption of sugars and urea were analyzed. Fed –batch studies were also carried out. Glucose and fructose were consumed during the exponential growth phase and were depleted after 18 hour of growth. On the contrary, *Candida sake* cells assimilated sucrose during the stationary phase and no growth improvement was seen by using fed batch technique. Addition of an extra amount of glucose or molasses after 18 hours of growth did not contribute to increase final population.

MATERIAL AND METHODS

Bacterial strain

A plant growth promoting nitrogen fixing *Rhizobium* strain BIHB 645 obtained from the culture repository of Plant Pathology and Microbiology Laboratory, Institute of Himalayan Bioresource Technology was used in the present studies.

Characterization of the bacterial strain:

Characterization of bacterial strain was done on the basis of morphological features, biochemical tests, carbon-source utilization pattern, fatty acid methyl ester analysis and 16S rRNA gene sequencing.

Morphological characterization:

Following tests were performed to characterize the bacterial strain morphologically:

a) Colony morphology:

Characteristics such as shape, margin, elevation and pigment colour were observed on yeast mannitol agar medium.

b) Gram reaction:

Thin homogenous bacterial smears were prepared from 1 d-old broth culture on clean glass slides, air dried, and heat fixed. The smear was covered with crystal violet stain for 1min, washed with distilled water, and flooded with mordant Gram's iodine solution for 1 min. The slide was washed with distilled water and decolorized with absolute alcohol until no violet colour came off. The smear was counter stained with 0.5% safranin (Appendix II) for 30 sec. The smear was washed, blot dried and observed under microscope using oil immersion objective. Gram positive bacteria appeared bluish purple and Gram negative bacteria appeared pinkish red in color.

c) Motility test:

The motility test was performed following the Hanging Drop method. Petroleum jelly was applied to the edges of a cleaned cover slip. A loop of 1 d-old culture suspension in broth was transferred to the centre of the cover slip. A cavity was placed over the cover slip, with the cavity facing downwards, covering the suspension. Slide was gently pressed to seal up with the cover slip and turned over quickly. The preparation was observed under low and high power objectives. The bacteria that moved swiftly across the microscope field were connoted positive for motility.

Biochemical Characterization:

a) Catalase test:

A drop of 3% H_2O_2 (Appendix III) was added to the inoculum taken on a glass slide. Appearance of effervescence in H_2O_2 drop indicated positive catalase activity.

b) Oxidase test:

A speck of culture was rubbed to Oxidase disc soaked with pure sterilized water and placed over a clean slide. Appearance of the dark purple colour within 10 to 20 sec indicated positive Oxidase activity.

d) Indole production:

Peptone broth was inoculated with the bacterial culture and incubated for 24 h at 28 ^{o}C. After the incubation of 24 h at 28 ^{o}C, 0.5 ml of Kovac's reagent (p-dimethyleaminobenzaldehyde) was added and observed for indole production. Appearance of deep golden red ring indicated positive reaction.

e) Citrate utilization:

Slants of Simmon's citrate agar (Appendix I) were inoculated with the bacterial culture and incubated for 48-72 h at 28 ^{o}C. Positive test is indicated by increase in pH of agar, changing Bromo thymol Blue indicator in the medium from green to deep blue.

f) O-F test:

Inoculated the bacterial culture in two tubes containing O-F medium (Appendix I) with added carbohydrate (dextrose). Added 2 ml of paraffin liquid light in one tube and incubated both for 48 h at 28 ^{o}C. Fermentative bacteria changed color to yellow in both tubes. Oxidative bacteria changed color to yellow in the tube without paraffin.

g) Urease test:

Slants of Christensen's medium (Appendix I) containing urea were inoculated with the bacterial culture and incubated at 28 ^{o}C. Examined the tubes after 4 h and 24 h. Positive test is indicated by a change in colour from yellow-orange to red-violet in 1-4 days.

h) Methyl Red and Voges-Proskauer (MRVP) test:

5 ml of MRVP medium was poured into tubes and the cultures were inoculated in two tubes. Tubes were incubated for 18-24 hrs at 28 ^{o}C. 2-3 drops of methyl red indicator solution and 600µl each of reagent A and B were added for MR test and VP test respectively. Positive reaction is indicated by red and pink-red colouration for MR test and VP test respectively.

Carbon source utilization pattern

Carbohydrate utilization pattern for 95 different carbon sources by bacterial isolate was studied using BIOLOG system (BIOLOG Microstation^{TM}) and BIOLOG Microlog^{TM} version 4.20.05 software. Culture grown on trypticase soya agar (TSA, Appendix I) for 48 h at 28 °C were re-suspended in 20 ml inoculation fluid (BIOLOG) and inoculum density adjusted to 52% transmittance using BIOLOG Turbidimeter. BIOLOG GN microplate inoculated with

150 µl of cell suspension per well were incubated at 30 °C. The plate was read at 595 nm using MicroStation Reader between 4-6 h and 16-24 h of incubation. Substrate utilization profiles were compared with MicroLog 4.2 database software. The results were scored as negative (-) or positive (+).

Whole-cell fatty acids methyl ester (FAME) analysis:

The bacterial strain was identified based on whole-cell fatty acids derivatized to methyl esters and gas chromatographic (GC) analysis using Sherlock Microbial Identification System (MIDI, Inc. Newark, DE, USA).

Harvesting of cells: Bacterial isolate was grown on trypticase soya agar (TSA) at 28 °C for 24 h. Took 3-4 loops of bacterial cells from the third quadrant of the quadrant streaked plates in 13 mm×100 mm screw capped vials for saponification.

Saponification: Added 1.0 ml of Reagent 1 (Appendix II) to each vial containing bacterial cells, sealed the vials with teflon-lined caps, vortexed 5-10 sec and heated in a boiling water bath for 30 min with 5-10 sec vigorous vortexing every 5 min.

Methylation: Added 2 ml Reagent 2 (Appendix II) to the cooled vials, vortexed 5-10 sec, and heated at 80 °C in a water bath for 10 min. The contents were cooled quickly by placing the vials in ice.

Extraction: Added 1.25 ml Reagent 3 (Appendix II) to the cooled vials followed by gentle tumbling for 10 min on a rotator. The two layers were separated and the lower aqueous layer discarded.

Base wash: Added 3 ml Reagent 4 (Appendix II) to the vials containing the organic phase. The vials were rotated end-over-end for 5 min and centrifuged for 5 min at 3000 rpm. The upper solvent phase was pipetted into GC vials.

Gas chromatography: The samples were subjected to GC analysis on GC 6890 (Agilent Technologies, USA) using 25 m×0.2 mm phenyl methyl silicone fused silica capillary column. Hydrogen was used as the carrier gas, nitrogen as the "make up" gas and air to support the flame. The GC oven temperature was programmed from 170 to 270 °C at 5 °C per min, with 2 min hold at 300 °C.

Fatty acids were identified and quantified by comparison to retention time and peak area obtained for the authentic standards. Qualitative and quantitative differences in the fatty acid profile was used to compute the distance for the strain BIHB 645 relative to the strains in the Sherlock bacterial fatty acid reference libraries TSBA50 5.00 and CLIN50 5.00 meant for aerobes.

Genomic DNA isolation:

Genomic DNA was extracted using Qiagen DNeasy® Plant Mini kit (Qiagen, CA) according to the manufacturer's instructions. The bacterial strain grown overnight in 10 ml NB were pelleted at 10,000 rpm for 10 min at 4 °C using a Refrigerated Centrifuge 3K30 (Sigma, CA). The pellets were suspended in 400 µl Buffer AP1 and 4 µl of RNase A stock solution (100 mg/ml), vortexed for 2-3 sec, incubated at 65 °C for 10 min and mixed 2-3 times during incubation by inverting the tube. Added 130 µl of Buffer AP2 to the lysate, mixed and centrifuged at 14,000 rpm for 5 min. Applied the lysate to QIAshredder Mini Spin Column placed in a 2 ml collection tube and centrifuged at 14,000 rpm for 2 min. Transferred the flow-through to a new tube without disturbing the cell-debris pellet. Added 675 µl (1.5 volumes) of Buffer AP3/E to the cleared lysate, mixed by pipetting and applied 650 µl to DNeasy Mini Spin Column placed in a 2 ml collection tube. Centrifuged at 8,000 rpm for 1 min and discarded the flow-through. Added the remaining sample to the column, centrifuged at 8,000 rpm for 1 min and discarded the flow-through. Added 500 µl Buffer AW to DNeasy

Mini Spin Column placed in a new 2 ml collection tube and centrifuged for 1 min at 8,000 rpm. Discarded the flow-through and added 500 µl Buffer AW to DNeasy Mini Spin Column and centrifuged for 2 min at 14,000 rpm. Transferred the DNeasy Mini Spin Column to a 1.5 ml microcentrifuge tube and pipetted 100 µl Buffer AE directly onto the DNeasy membrane. Incubated for 5 min at 15-25 °C and centrifuged for 1 min at 8,000 rpm to elute the genomic DNA. The quality of DNA was checked on 0.75% agarose gel prepared in 1× TAE buffer (Appendix III).

16S rRNA sequence analysis:

16S rRNA gene amplification was performed using the universal primers fD1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rP2 (3'-ACG GCT ACC TTG TTA CGA CTT-5') (Weisburg *et al*., 1991). The total volume of 50 µl PCR reaction mixture was comprised of 200 µM dNTPs, 50 µM each primer, 1× PCR buffer, 3 U Taq polymerase, and 100 ng genomic DNA. The thermocycling procedure involved an initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min, and final extension at 72 °C for 8 min. The PCR products were analyzed on 1.2 % agarose gel and their size estimated using 1 kb DNA ladder (Fermentas, Vilnius).

The amplified DNA band corresponding to ~1500 bp was excised using a sharp blade and eluted according to the manufacturer's instructions employing PureLink^{TM} Quick Gel Extraction Kit (Invitrogen, CA). Placed nearly 400 mg gel into a 1.5 ml polypropylene tube, added 1.2 ml Gel Solubilization Buffer (GS1), incubated at 50 °C for 15 min and mixed every 3 min to ensure gel dissolution. After the gel dissolution incubated for an additional 5 min. Placed Quick Gel Extraction Column into 2 ml Wash Tube and loaded the dissolved gel piece onto the column, centrifuged at 12,000 *g* for 1 min, discarded the flow-through and placed the column back into the Wash Tube. Added 500 µl Buffer GS1 to the column,

incubated at room temperature for 1 min, centrifuged at 12,000 *g* for 1 min. Discarded the flow-through and placed the column back into the Wash Tube. Added 700 µl Wash Buffer (W9) with ethanol to the column, incubated at room temperature for 5 min, centrifuged at 12,000 *g* for 1 min and discarded the flow-through. Centrifuged at 12,000 g for 1 min to remove any residual Wash Buffer (W9), and discarded the Wash Tube. Placed the column into a 1.5 ml Recovery Tube, added 50 µl warm (60-70 °C) TE Buffer to the column center, incubated for 1 min at room temperature, centrifuged at 12,000 *g* for 2 min, discarded the column and stored the purified 16S rRNA gene product at -20 °C.

The purified 16S rRNA gene was cloned into pGEM-T easy vector and transformed to chemically competent *Escherichia coli* JM 109 cells following the manufacturer's protocol (Promega, Madison). The transformation mix was incubated for 3 h at 37 °C. 100 µl of the transformation culture was plated on Luria-Bertani (LB) agar containing 20 µl of 5-bromo-4-chloro-3-indoyl-ß-D-galactopyranoside (X-Gal, Appendix II), 100 µl of 0.1 M isopropyl-β-D-1-thiogalactopyranoside (IPTG, Appendix II) and 100 µg ml^{-1} ampicillin. The white colonies appearing after overnight incubation at 37 °C were transferred to fresh LB agar plates amended with 100 µg ml^{-1} ampcillin.

Cloning of 16S rRNA gene was confirmed by colony PCR. The PCR reaction mix consisted of 2.5 µl 10× PCR buffer, 1µl of 200 µM dNTPs, 1µl of 50 µM of each primer, 0.5µl of 3U *Taq* DNA polymerase (Invitrogen, CA) and single bacterial colony employed as the template. The thermocycling procedure involved an initial denaturation at 96 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 2 min, and final extension at 72 °C for 8 min. The PCR product was resolved on 1.2 % agarose gel. The clones showing single amplicon of 1500 bp were employed for plasmid DNA isolation. Recombinant plasmids were purified by using Plasmid Gene Elute Miniprep Kit (Sigma,

CA). Overnight grown recombinant *E. coli* culture in 2 ml LB was pelleted by centrifugation at 10,000 rpm for 10 min at 4 °C. The pellet was suspended in 200 µl resuspension solution by pipetting up and down until the suspension was homogenous. The cells were lysed by adding 200 µl lysis solution. The contents were mixed by gentle inversion 6-8 times until the mixture became clear and viscous. The cell debris was precipitated by adding 350 µl of neutralization solution by inverting the tubes 4-6 times and pelleted by centrifugation at 12,000 rpm for 10 min. A Gene Elute Miniprep Binding column was inserted into a microfuge tube and 500 µl of column preparation solution added to each miniprep column, centrifuged at 12,000 rpm for 1 min, and flow through discarded. 500 µl optional wash solution was added to the column, centrifuged at 12,000 rpm for 1 min, and flow through discarded. The column was washed by adding 750 µl of diluted wash solution to the column. The flow through liquid was discarded and centrifuged again at maximum 12,000 rpm for 2 min without any additional wash solution to remove excess ethanol. The column was transferred to a fresh collection tube and plasmid DNA eluted by adding 100 µl nuclease free water (18.2 Ω) to the centre of column matrix, followed by centrifugation at 12,000 rpm for 2 min. The quality of purified plasmid DNA was checked on 1% agarose gel prepared in 1× TAE buffer.

Sequences of the insert were determined using Big-Dye Terminator Cycle Sequencing Mix (Applied Biosystems, CA). The sequencing PCR reaction of 5 µl included 1 µl 5× sequencing buffer, 1 µl Big-Dye Terminator premix, 1 µl primer (5 pmol) and 2 µl purified plasmid DNA. Thermal cycling conditions consisted of an initial denaturation at 96 °C for 3 min, followed by 30 cycles of 94 °C for 10 sec, 50 °C for 40 sec and 60 °C for 4 min. The unincorporated dye terminators were removed using Montage SEQ_{96} Sequencing Reaction Clean up Kit (Mllipore, MA). The purified sequencing products were transferred to 96 well

injection plates. The sequences were elucidated on 3130xGenetic Analyzer (Applied Biosystems, CA). The sequences obtained were searched as query to the BLASTn http://www.ncbi.nih.gov search algorithm. The culture has been deposited in the Microbial Type Culture Collection and Gene Bank at the Institute of Microbial Technology, Chandigarh, India.

Optimization of media ingredients for high cell density cultivation of *Rhizobium* strain BIHB 645

Selection of most significant medium ingredients affecting growth using 'one variable at a time' approach

The effect of yeast mannitol broth (Appendix 1) ingredients on the biomass production of *Rhizobium* strain 645 was studied by removing ingredients one by one from YMB as follows:

S. No.	Medium	S. No.	Medium
1	YMB	6	YMB without yeast extract
2	YMB without K_2HPO_4	7	YMB with 2.5 g/l mannitol
3	YMB without $MgSO_4$,	8	YMB with 5 g/l mannitol
4	YMB without NaCl	9	YMB with 7.5 g/l mannitol
5	YMB without $CaCO_3$		

Fifty milliliter media in 250 ml Erlenmeyer flasks were inoculated with 250 µl of 48 h old YMB culture and incubated at 28 ºC for 60 h at 180 rpm in a refrigerated incubator shaker (Innova Model 4230, New Brunswick Scientific, USA). The serial dilutions 10^{-2} to 10^{-8} of culture growth were spread plated on yeast mannitol agar (Appendix 1) plates at 6 h intervals up to 60 h of incubation. The plates were incubated at 28 ºC to determine the viable colony forming units per ml of the media.

Optimization of culture growth conditions using Box-Behnken Design of Response Surface Methodology

The most limiting ingredients of YMB along with temperature were optimized to develop a mathematical correlation between three independent variables on the biomass production of *Rhizobium* strain BIHB 645 statistically by Response Surface Methodology (RSM) using the Box-Behnken design (Box and Behnken, 1960). The models were studied using a range of variables at three different levels designated as low (-1), middle (0) and high (+1) concentrations:

		Levels (g/l)		
Factor code	**Factor**	**-1**	**0**	**+1**
X_1	Temperature (^oC)	19	28	37
X_2	Mannitol	2.5	5	7.5
X_3	Yeast extract	0.25	1	1.75
	K_2HPO_4	-	0.5	-
	$MgSO_4$	-	0.2	-
	Nacl	-	0.1	-
	$CaCO_3$	-	1.0	-

Mannitol was replaced with molasses in another set of experiment as follows:

Factor code	Factor	Levels (g/l)		
		-1	**0**	**+1**
X_1	Temperature	19	28	37
X_2	Yeast extract	0.25	1.0	1.75
X_3	Molasses	5	10	15
	K_2HPO_4	-	0.5	-
	$MgSO_4$	-	0.2	-
	NaCl	-	0.1	-
	$CaCO_3$	-	1.0	-

Biomass was taken as response (G) and a multiple regression analysis was done for obtaining an empirical model which related the response measured to the independent variables. The behavior of the system was explained by the following quadratic model equation:

$$G = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

where, G is the predicted response, β_0 intercept, β_1, β_2, and β_3 linear coefficients, β_{11}, β_{22} and β_{33} squared coefficients and β_{12}, β_{13} and β_{23} interaction coefficients. The MINITAB Inc., version 11.12, PA, USA statistical software was used to obtain optimal working parameters and to generate response surface graphs.

A 2^3 factorial design for independent variables yeast extract, mannitol and temperature each at three levels with five replicates at the centre point leading to 17 runs was employed to optimize the medium ingredients:

Run No	Medium Composition (g/l)						
	Temperature	Mannitol/ Molasses	Yeast extract	K_2HPO_4	NaCl	$MgSO_4$	$CaCO_3$
1	0	-1	-1	0	0	0	0
2	0	+1	-1	0	0	0	0
3	0	-1	+1	0	0	0	0
4	0	+1	+1	0	0	0	0
5	-1	-1	0	0	0	0	0
6	-1	+1	0	0	0	0	0
7	+1	-1	0	0	0	0	0
8	+1	+1	0	0	0	0	0
9	-1	0	-1	0	0	0	0
10	-1	0	+1	0	0	0	0
11	+1	0	-1	0	0	0	0
12	+1	0	+1	0	0	0	0
13	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0

Method:

The flasks containing 50 ml media were inoculated with 250 µl of 48 h old culture grown in YMB medium and incubated at 19, 28 and 37 ºC for 54 h at 180 rpm in a refrigerated incubator shaker. The serial dilutions from 10^{-2} to 10^{-8} of each sample were spread plated on YMA plates and incubated at 28 ºC for 48 h to determine the viable colony forming units.

Biomass determination was done by centrifuging 1 ml culture aliquots at 13,000 rpm for 10 min in preweighed Eppendorf tubes, pellets washed twice with normal saline, dried in a vacuum spin concentrator (Thermo Electron Corporation, Milford, MA) and weighed. Viable cells were expressed as CFU/ml and biomass as g/l.

Software and data analysis

Statistical software package 'Design-Expert® 6.0' Stat-Ease Inc. (Minneapolis, MN, USA) was used for generating the experimental design and statistical analysis of the data. The optimal concentrations of the medium components were obtained by analyzing the response surface contour plots. Fisher's F-test was used for statistical analysis of data and determination coefficient R^2 for the goodness of fit of regression model.

Validation of the experimental model

Validation of the model and regression equation was performed by taking the optimum values of mannitol, yeast extract and temperature for biomass determination in 50 ml medium in 250 ml shake flasks with five replicates:

Growth conditions	Medium	
	I	**II**
Mannitol (g/l)	5.5	-
Molasses (g/l)	-	15
Yeast extract (g/l)	1.7	1.00
KH_2PO_4 (g/l)	0.50	0.50
$MgSO_4$ (g/l)	0.20	0.20
NaCl (g/l)	0.10	0.10
$CaCO_3$ (g/l)	1.00	1.00
Temperature (ºC)	28	28

Up-scaling Inoculum Production

Fermentation experiments for up-scaling inoculum production was performed in a AD1 1065 Bio Bench Reactor Systems with AD1 1010 Bio Controller and AD1 1025 Bio Console (Applikon Dependable Instruments, AE Schiedam) bioreactor with total volume of 20 l and working volume of 14 l. BioXpert Version 1.2x, 2001 software (Applikon Dependable Instruments, AE Schiedam) was used to implement all supervisory control tasks. The optimized media composition and temperature as previously described was used for batch fermenter experiments. The culture media was inoculated with 0.5% of the preculture prepared by growing the bacterium in YMB medium in an incubator shaker at 28 ºC for 48 h at 180 rpm. The pH was maintained at 7.0 by controlled addition of 1 N NaOH. Antifoam (A6758, Sigma) was added automatically when required. Air flow was kept constant at 10 l min^{-1}. The concentration of dissolved oxygen (DO) was kept constant at 20% saturation by automatically adjusting stirrer speed.

RESULTS

Phenotypic characterization

The colony morphology of *Rhizobium* BIHB 645 was circular, convex, smooth, slimy, white and translucent with entire margins. The isolate was Gram-negative, motile rods, positive for oxidase and VP but negative for indole, MR, catalase, utilization of dextrose in O-F medium and urease (Fig. 1a-c).

Carbon-source utilization pattern

The bacterial isolate was positive for utilization of L-arabinose, dextrin, putrescine, glycerol, 2-amino ethanol, ß-methyl D-glucoside, D-galactonic acid, lactone, D-alanine and urocanic acid but negative for utilization of α-D-glucose, cis-aconitic acid, citric acid, D-gluconic acid, formic acid, α-keto glutaric acid, DL lactic acid, propionic acid, quinic acid, bromo succinic acid, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, hydroxyl L-proline, L-proline, L-pyroglutamic acid, L-serine, γ-amino butyric acid, D-arabitol, D-galactose, m-inositol, mannitol, mannose, D-glucosaminic acid, D-glucuronic acid, ß-hydroxy butyric acid, malonic acid, D-saccharic acid, succinic acid, succinamic acid, L-alanyl glycine, L-leucine, L-ornithine, inosine L-rhamnose, D-sorbitol, sucrose, mono-methyl-succinate, methyl pyruvate, D-galacturonic acid α-cyclodextrin, glycogen, tween 40, Tween 80, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, cellobiose, erthyritol, L-Fucose, D-Fructose gentiobiose, α-D-lactose, lactulose, maltose, melibiose, D-psicose, , D-raffinose, turanose, D-trehalose, xylitol, acetic acid, α-hydroxy butyric acid, γ-hydroxy butyric acid, itaconic acid, p-hydroxy phenylacetic acid, α-keto butyric acid, α-keto valeric acid, sebacic acid, glucuronamide, alaninamide, glycyl-L-aspartic acid, glycyl-L-

glutamic acid, phenyl alanine, D-serine, L-threonine, DL-carnitine, uridine, thymidine, phenyl ethylamine, 2,3-butanediol, DL-α-glycerol phosphate glucose-6-phosphate and glucose-1-phosphate (Table 1). The carbon-source utilization pattern showed highest similarity of 0.21 of the isolate with *Burkholderia glumae.*

Fatty acids methyl ester (FAME) analysis

The composition of cell-wall fatty acids of the strain is presented in the Table 2. The fatty acids 16:0, 18:0 and 18:1 w7c 11-methyl were present in the strain (Fig. 2). Small amount of 15:0 2OH was also detected. The analysis showed the highest similarity of the strain with *Methylobacterium-aminovorans*

16S rRNA gene sequencing

Purified total genomic DNA of nitrogen fixing Rhizobium strain used as template generated a single band of approx. 1500 bp amplification product (Fig. 3). The single band of approx. 1500 bp in the colony PCR of randomly picked white colonies on LB agar confirmed the transformation of 16S rRNA gene into *Escherichia coli* JM 109 (Fig. 4-7). The two 3 kb bands obtained by resolving plasmid DNA harvested from positive clones on agarose gel confimed cloning of the recombinant DNA (Fig. 8). Each of the forward (T7) and reverse (SP6) primers was able to elucidate approx. 900 bp sequence of the recombinant plasmid DNA. The results of BLAST search of 16S rDNA sequences of the strain on comparison with the available 16S rRNA gene sequences in the GenBank database indicated 99.9% homology of the strain with *Rhizobium leguminosarum* ATCC 14480. The 16S rRNA gene sequence has been submitted with NCBI GenBank with the accession number DQ 536522.

Optimization of medium ingredients for high cell density cultivation of *Rhizobium* strain BIHB 645

Selection of the medium ingredients affecting growth by 'one variable at a time' approach

The results on the effect of different YMB ingredients on the biomass production of *Rhizobium leguminosarum* BIHB 645 are shown in Fig. 9. The strain BIHB 645 exhibited maximum growth of 9.3×10^{10} CFU ml^{-1} in YMB with 2.5 g/l mannitol by 54 h followed by 6.8×10^{10} CFU ml^{-1} in YMB with 5g/l mannitol by 54 h, 5.9×10^{10} CFU ml^{-1} in YMB with 7.5g/l mannitol by 54 h, 4.5×10^{10} CFU ml^{-1} in YMB with 2.5 g/l mannitol by 54 h, 3.4×10^{10} CFU ml^{-1} in YMB without NaCl by 54 h, 2×10^{10} CFU ml^{-1} in YMB without $MgSO_4$ by 54 h, 7.8×10^{9} CFU ml^{-1} in YMB without K_2HPO_4 by 54 h, 6×10^{9} CFU ml^{-1} in YMB without $CaCO_3$ by 42 h, and 5.4×10^{7} CFU ml^{-1} in YMB without yeast extract by 18 h of incubation. The most limiting factor affecting the growth was yeast extract followed by mannitol. The removal of K_2HPO_4, $MgSO_4$, NaCl and $CaCO_3$ from the medium one at a time did not affected biomass production of the strain BIHB 645. Therefore, yeast extract and mannitol were selected as the limiting growth factors for further studies.

Box-Behnken Design of Response Surface Methodology for high cell density culture of *Rhizobium* sp 645

Growth medium with mannitol as a carbon source:

Following the results of 'one variable at a time' approach the effect of yeast extract and mannitol on the growth of *Rhizobium* sp. BIHB 645 was studied at three different temperatures. The results of RSM experiment for studying the effect of yeast extract, mannitol and temperature, with the actual and predicted responses are presented in Table 3. The coefficient of determination (R^2) was calculated to be 96.9%, which showed that the model correlated well with measured data and was statistically significant at $p \leq 0.005$ (Table

4). The data showed that all the three variables in one dimension play a significant role in biomass production at p values <0.5. The regression analysis showed that the selected independent variables temperature, mannitol were significant at $p \leq 0.005$ for the biomass production in linear as well as in squared terms (Table 4). The interaction between mannitol and yeast extract was also significant at $P \leq 0.5$. However, the negative coefficients showed the negative interaction between the variables. The contour plot 10 showed that maximum biomass of 2.0 g/l was obtained in the medium containing 5.5 g/l mannitol; 1.7 g/l yeast extract, 0.50 g/l K_2HPO_4; 0.20 g/l $MgSO_4$; NaCl 0.10g/l; $CaCO_3$ 1.0g/l at 28 ºC. The experiment was repeated under above conditions to validate the results (Table 6). The experimental values for biomass (1.86±0.065) were closer to the predicted values, thus validating the model.

Growth medium with molasses as the carbon source:

The results of RSM experiment for studying the effect of temperature, molasses and yeast extract with the actual and predicted responses are presented in Table 5. The coefficient of determination (R^2) was calculated to be 97.7%, which showed that the model correlated well with measured data and was statistically significant at $p \leq 0.005$ (Table 5). The regression analysis showed that among the three variables, yeast extract and temperature were significant at $p \leq 0.005$ for the biomass production in linear as well as in square terms. The interaction between yeast extract and temperature played a significant role in biomass production at p values <0.005. However the negative coefficients showed a negative interaction between the two variables. The contour plot 11 showed maximum biomass of 2.30 g/l when the molasses concentration was 15 g/l , yeast extract concentration was 1.0 g/l , $MgSO_4$ concentration was 0.20 g/l, K_2HPO_4 concentration was 0.50 g/l, $CaCO_3$ concentration was 1.0 g/l. So, the experiment was repeated under above conditions to

validate the results (Table 6). The experimental values for biomass (2.2±0.057) were closer to the predicted values, thus validating the model.

Upscaling

The effect up of agitation rate on the scaled up production of biomass for *Rhizobium leguminosarum* BIHB 645 was studied in a Bio Bench bioreactor at 28 ºC temperature and pH 7.0 for 26 h (Fig. 12). The results showed that highest biomass of 5.0 g/l was observed at 300 rpm by 24 h of incubation (Fig. 13). The biomass obtained in bioreactor studies was two fold higher than that attained in shake flasks in optimized medium.

DISCUSSION

Symbiotic and free-living bacteria can influence plant growth and yield in a number of ways. Rhizobium is aggressive root colonizers of leguminous and some non-leguminous plants, capable of promoting crop yield. A plant growth promoting nitrogen fixing *Rhizobium* strain isolated from the root nodules of *Pisum sativum* was characterized on the basis of polyphasic approach. Morphological features and biochemical tests showed the strain belonging to genus *Rhizobium.* The nitrogen fixing associations of rhizobia with leguminous plants are of great importance both in global nitrogen cycling and in agriculture (Evans *et al*., 1991; Postage 1992; Somasegaran and Hoben, 1994). Polyphasic approach including genotypic, chemotaxonomic and phenotypic methods has been employed by various workers for identifying and characterizing bacteria (Chung *et al.* 2005; Gulati *et al*., 2008; Gulati *et al*., 2009). The results of the present studies that the strain could not be identified on the basis of carbon source utilization pattern corroborated the earlier study on characterization of a plant growth-promoting and rhizosphere-competent strain where the strain could also not be identified based on carbon source utilization pattern (Gulati *et al*., 2009). FAME analysis showed 20.0% similarity of the strain BIHB 645 with *Methylobacterium-aminovorans* while 16S rRNA gene sequencing confirmed the strain to be *Rhizobium leguminosarum*. The results substantiated the earlier reports on characterization based on polyphasic approach that identification based on carbon source utilization pattern, FAME analysis and 16 S rRNA gene sequencing does not necessarily be same. The isolates showing similarity with *Pseudomonas putida* on the basis of FAME analysis showed more than 99% identity with *Pseudomonas trivialis*, *P. poae* and *Pseudomonas* sp. (Gulati *et al*., 2008). Similarly, a plant growth-promoting and rhizosphere-

competent strain could not be identified based on carbon source utilization pattern but identified as *Acinetobacter calcoaceticus* on the basis of FAME analysis and 16S rRNA gene sequencing confirmed the isolate to be *Acinetobacter rhizospaerae* (Gulati *et al*., 2009). Similar results were obtained by Chung *et al.* (2005) while characterizing phosphate-solubilizing bacteria on the basis of FAME analysis and 16S rRNA gene sequencing from the rhizosphere of crop plants of Korea.

High cell density cultures offer an efficient means for the economical production of various products. As mentioned by Lee (1996) and Reisenberg and Guthke (1999) increasing productivity is the major goal of fermentation in research and industry and so HCDC are a prerequisite to achieve this goal. In general, no defined medium has yet been established for mass production of any PGPR strain for commercial purpose. Each organism has its own requirement for maximum biomass production (Elibol, 2004). The basal medium for growth of *Rhizobium* was yeast mannitol broth. The selection of a most significant medium ingredients which affect the growth is necessary to increase the biomass production and to further optimize culture growth conditions for *Rhizobium leguminosarum* BIHB 645 so that commercial production process could be evolved. The results revealed that mannitol and yeast extract had the most profound effect on *Rhizobium* growth and therefore these factors along with temperature were selected for statistical optimization by RSM. These observations clearly indicate the relationship between culture conditions and biomass production.

The results on the optimization of culture growth conditions using Box-Behnken design of Response Surface Methodology revealed that the model used was good fit with Rsq value of 97.7 % when growth medium with molasses used as a carbon source. A higher value of correlation coefficient, justifies an excellent correlation between the

independent variables. The data clearly demonstrated that the concentrations of molasses, yeast extract and temperature have a direct relationship with biomass production. The linear and quadratic effects of temperature, yeast extract, molasses and interaction effect of yeast extract and temperature were highly significant. This implies that they can act as limiting factors and minor variation in their concentration may affect the rate of biomass production.

Response surface plots are the graphical representation of regression equations and so they provide us a method to visualize the interaction of independent variables for maximum biomass production (Murat Elibol, 2003). Each contour curve represent an infinite number of combination of 2 test variables with other maintained at their respective zero levels. In the present studies, the contour plot between yeast extract and molasses revealed that a biomass of 2.30 g/l (61.7% increase) could be obtained in a medium containing 15 g/l of molasses, 1.0 g/l of yeast extract, 0.50 g/l of K_2HPO_4, 0.20 g/l of $MgSO_4$, 0.10 g/l of NaCl and 1.0 g/l of $CaCO_3$ at 28 $^{\circ}$C which was higher than the biomass produced in the presence of mannitol as the carbon source (2.0 g/l). The validation was carried out in shake flasks under optimum conditions of the media predicted by the model. Validation of the model suggested unequivocally, the reliability of RSM for optimization of culture conditions for maximum biomass production of *Rhizobium leguminosarum* strain BIHB 645. By using the optimized batch process biomass production increased gradually with the progression of fermentation and reached upto 5 g/l after 24 hours, as compared to 2.3g/l after 54 hours in shake flasks, and declined slightly thereafter. These results substantiate with the results reported by Kaur and Satyanarayna, (2004) where 1.7 fold increase in biomass production of *Thermomucor indicae-seudaticae* was achieved in 40 hour instead of 48 hour in shake flasks. High biomass production is generally expected in fermenter because of the better control of the process

parameters including improved aeration, proper mixing of nutrients and maintenance of pH (Kaur and Satyanarayna, 2005).

SUMMARY

Rhizobia include a wide range of bacteria capable of eliciting nitrogen-fixing nodules in leguminous plants. These nitrogen-fixing soil bacteria, which directly or indirectly stimulate the plant growth, have been targeted as the potential plant growth promoting bacteria. Plant growth promoting bacteria (PGPB) offer an alternative to the use of inorganic fertilizers and chemical pesticides in enhancing crop productivity. Use of PGPB as microbial inoculant to increase agricultural production, biocontrol of plant pathogens and an aid in bioremediation is getting world wide attention. Identification and characterization of beneficial microorganisms is important to develop deployment strategies for increasing their contribution in agriculture. A plant growth promoting nitrogen-fixing *Rhizobium* strain isolated from the root nodules of *Pisum sativum* growing in the cold deserts of the trans-Himalayas was characterized on the basis of morphological features, carbon-source utilization pattern, whole cell fatty acids methyl ester analysis and 16S rRNA gene sequencing. The bacterial strain couldn't be identified on the basis of carbon source utilization pattern as it showed highest similarity index of 0.21 with *Burkholderia glumae* available in the BIOLOG database. FAME analysis showed 20.8% similarity of the strain with *Methylobacterium aminovorans*. The results of BLAST search of 16S rRNA sequences of the strain on comparison with the available 16S rRNA gene sequences in the GenBank database indicated 99.9% homology of the strain with *Rhizobium leguminosarum* ATCC 14480.

The medium constituents and the process parameters were optimized by single factor optimization keeping the other factors constant. It was found that mannitol and yeast extract had the most profound effects on *Rhizobium* growth and therefore these factors along with temperature were selected for statistical optimization by RSM so that the production of biomass could be increased. In order to search for the optimum combination of mannitol, yeast extract and temperature, experiments were performed using Box-Behnken design of Response Surface Methodology. The optimal culture growth conditions for *Rhizobium leguminosarum* composed of (g/l): molasses 15.0; yeast extract 1.0; K_2HPO_4 0.50; $MgSO_4$ 0.20; NaCl 0.10; $CaCO_3$ 1.0 at 28 ºC. Under the optimal conditions, maximum biomass produced was 2.3 g/l, with an increase of 61.7% compared to original medium. The biomass production in the optimized medium was in good agreement with the values predicted by the Box Benkehn design of response surface methodology and was sustainable in fermenter as it attained a peak within 24 hours as compared to that of 54 hours in shake flasks. The present study highlighted the importance of optimizing culture variables for attaining improvement in biomass production. As PGPR reveal huge potential for crop production, for microbial inoculants to be commercially feasible, it must be economically mass-produced and formulated into a cost-effective, uniform and readily applicable form.

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Appendix I

Culture Media

Nutrient broth (g/litre):

Peptic digest of animal tissue	5.0
NaCl	5.0
Beef extract	1.5
Yeast extract	1.5
pH	7.0 ± 0.2

Nutrient agar (g/litre):

Added 18.0 g agar agar to 1 litre nutrient broth

Trypticase soya broth (g/litre):

Pancreatic digest of casein	17.0
Papaic digest of soyabean meal	3.0
NaCl	5.0
K_2HPO_4	2.5
Dextrose	2.5
pH	7.3 ± 0.2

LB broth (g/litre):

Tryptone	10.0
Yeast extract	5.0
NaCl	5.0
pH	6.8±0.2

LB agar (g/litre):

Tryptone	10.0
Yeast extract	5.0
NaCl	5.0
Agar agar	15.0
pH	6.8±0.2

Yeast Mannitol broth (g/litre):

Mannitol	10.00
Yeast extract	1.00
K_2HPO_4	0.50
$MgSO_4$	0.20
NaCl	0.10
$CaCO_3$	1.00
pH	6.8±0.2

Yeast Mannitol Agar (g/litre):

Added 15.0 g agar agar to 1 litre Yeast Mannitol broth

Christensen's Medium(g/litre):

Peptone	1g
NaCl	5g
KH_2PO_4	2g
Phenol red	6ml
Agar	20g
D.W.	1litre
pH	6.8-6.9

Simmons Citrate Medium (g/litre):

$MgSO_4.7H_2O$	0.2g
$NH_4H_2PO_4$	1g
K_2HPO_4	1g
$Na_3C_6H_7.2H_2O$	2g
NaCl	5g
Bromothymol blue	0.08g
Agar	15g
D.W.	1litre
pH	6.8

Methyl Red and Vagus-Proskaur Medium(g/litre):

Glucose	5 g
Peptone	5 g
K_2HPO_4	1.5 g
D.W.	1 litre
pH	7.5

Peptone Water Base(g/litre):

Peptone	10g
NaCl	5g

D.W.	1 litre
pH	7.2-7.3

OF Basal Medium(g/litre):

Casein enzyme hydrolysate	2g
Sodium chloride	5g
Dipotassium phosphate	0.30g
Bromothymol blue	0.08g
Agar	2g
D.W	1 litre
PH	6.8

Appendix II

Solutions and Reagents

Reagents required for the Fatty Acid Methyl Ester Analysis

Reagent 1 (Saponification):

Sodium hydroxide	45 g
Methanol (HPLC grade)	150 ml
Distilled water	150 ml

Reagent 2 (Methylation):

Hydrochloric acid (6N)	325 ml
Methanol	275 ml

Reagent 3 (Extraction Solvent):

Hexane (HPLC grade)	200 ml
Methyl Tert-Butyl Ether (HPLC grade)	200 ml

Reagent 4 (Base Wash):

Sodium hydroxide	10.8 g
Distilled water	900 ml

IPTG stock solution (0.1 M):

To 1.2 g IPTG added water to 50 ml final volume. Filter sterilized and stored at 4 °C.

X-Gal:

Dissolved 100 mg X-Gal in 2 ml N, N'-dimethyl-formamide. Covered with aluminium foil and stored at -20 °C.

Appendix III

Buffer

TAE buffer (50X) 1000ml:

Tris base	242.0g
Glacial acetic acid	57.1g
0.5M EDTA	100.0ml
pH	8.5