Abstract

Hydrogen is thought to be an ideal fuel for the future, which could not only arrest global warming but also protect ecological balance by eliminating the emission of gaseous pollutants in air. Now it is becoming a challenging problem for the researchers to find out an alternative means of H_2 production preferably for renewable energy sources. Biological hydrogen production has an edge over its chemical counterpart mainly because it is environmentally benign. Genetic approach has the potential not only to understand the fundamentals of biological H_2 production at molecular level but also to apply those concepts for the development of improved microbial strains for hydrogen production. *Enterobacter cloacae* IIT-BT 08 has been identified as a potential microbial strain for H_2 production. Several approaches have been explored to improve H_2 production using above strain such as redirection of biochemical pathways by conventional mutagenesis, improvement of bioreactor configuration *etc.* However genetic approach remianed untaped.

Six different sets of degenerate primers were designed and synthesized from the conserved domain of [Fe]-hydrogenase. A 750 bp of PCR fragment was amplified with a set of degenerate primers (designed from the conserved domain of *hydA* structural gene encoding for catalytic subunit of [Fe]-hydrogenase) using genomic DNA of *Enterobacter cloacae* IIT-BT 08 as template. This DNA fragment was cloned in pCR2.1TOPO-TA cloning vector and sequenced. The sequence showed the presence of an open reading frame (ORF) of 444 bp of calculated molecular mass of 16.1 kDa for encoded protein . The multiple alignment of the translated protein sequence showed significant similarity (40%) with the conserved zone (H-cluster) of [Fe]-hydrogenase from a wide range of hydrogen producing microbial strain. To assess the functional nature of the isolated *hydA* gene, ORF was sub-cloned in frame with GST in pGEX4T-1 prokaryotic expression vector and overexpressed in a non-hydrogen producing *E. coli* BL-21. IPTG induction of the transformed *E. coli* BL-21 cells showed the expression of a fusion protein of expected size (42.1 kDa). Recombinant protein was purified and western blot analysis showed that the protein was specifically recognized by anti-GST monoclonal antibody. Southern hybridization further confirmed the presence of this gene in *E. cloacae* IIT-BT 08 genome. *In-vitro* and *In-vivo* analysis proved that the isolated gene is catalytically active under anaerobic condition and

could also compliment H_2 production in a non-hydrogen producing strain of *E. coli.* A tentative molecular mechanism has been proposed for the H_2 production based on the analysis of the results obtained from the above experiment that the electron donor can donates the electron directly without the mediation of F-cluster.

The regulation of *hydA* gene expression was examined by RT-PCR using specific primers from the known sequence of *hydA* ORF. Aerobically grown cells of *E. cloacae* IIT-BT 08 showed the presence of a very faint transcription product. However, switching to anaerobic condition (3 h) induced the transcription of *hydA* rapidly as evident by RT-PCR, which led to H_2 production as analyzed by GC under anaerobic condition. This shows that *hydA* in *E. cloacae* IIT-BT 08 is transcriptionally regulated by anaerobic environment.

Initial biophysical characterization of the overexpressed *hydA* encoding protein was done by fluorescence spectroscopy, urea denaturation assay and CD spectroscopy. Intrinsic tryptophan fluorescence profile of the over expressed *hydA* protein revealed that it has an excitation maximum at 285 nm and emission maxima at 339 nm. The unfolding pattern of *hydA* encoding protein of *E. cloacae* IIT-BT 08 as a function of urea concentration showed that the mid point transition of unfolding occurs at 4 M urea. From the urea denaturation it is also evident that at 6 M urea the protein gets totally unfolded. Secondary structure as predicted from circular dichroism (CD) spectroscopy data using K2D software revealed that *hydA* from *E. cloacae* IIT-BT 08 has relatively high percentage of α-helix (48%) than β-sheet (11%).

To explore the possibility of commercial exploitation of recombinant *hydA*, different physico-chemical parameters like culture temperature (37°C) and pH 6 were found out for the maximization of H_2 production using recombinant *E. coli* under both batch as well as continuous H_2 production system. Rate of H_2 production was found to be two folds higher in continuous system as compared to batch system. How ever H_2 production was found to be less as compared to *E. cloacae* IIT-BT 08 which could be due to the large size of the GST tag protein or may be due to the lacking of the presence of other accessory gene along with the *hydA* ORF.

At the genetic level attempts has also been made to isolate the flanking region of the untranslated zone of *hydA* ORF that might have a role in transcriptional regulation of *hydA* expression under anaerobic condition, which requires further studies.