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Optimization of Arginine Deaminase Production from Indigenous Bacterium *Pseudomonas aeruginosa* PS2

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A B S T R A C T

Arginine deaminase (ADI) is an important anticancer drug worldwide used in the chemotherapy of arginine-auxotrophic tumors, such as hepatocellular carcinomas and melanomas. To date, ADI obtained from *Mycoplasma* has been commercially used in clinics. However, low yield, high toxicity, short proteolytic and low serum tolerances are the major limitations of clinically available ADI. In the present investigation, we have described optimization of environmental and nutritional requirements for maximum biosynthesis of ADI from bacterium *Pseudomonas aeruginosa* PS2. We observed that batch time 25 h, inoculum age 20 h, 8% (v/v) inoculum size, pH 6.5 and temperature 37 °C were found as the most suitable operating conditions for ADI production. Galactose, peptone, KH_2PO_4 and L-arginine were found as the best carbon, nitrogen, mineral ion and inducer for ADI production, respectively. These results suggested that *P. aeruginosa* PS2 could be used for large-scale production of ADI but further studies are still required for strengthening the current findings which are underway in our lab.

Introduction

A molecule of biological origin has great importance and plays a critical role in sustaining of life on earth. Proteins and peptides are the important class of biomolecules possesses diversified applications from the range of food and feed to pharmaceutical industry. Today, proteins and peptides based several pharmaceutics such as interferons, blood factors, thrombolytics, hormones, growth factors, antibodies, and enzymes are used as therapeutic agent for treatment of several life threatening diseases viz. cancer, diabetes, neurological disorder, coronary heart disease and HIV/AIDS. L-arginine deaminase (ADI) (E.C. 3.5.3.6) is an enzyme, extensively investigated as enzymatic based antineoplastic drug. As biocatalyst, ADI catalyses the irreversible hydrolysis of L-arginine to citrulline and ammonium (Wang and Li, 2014) and widely used as therapeutic agent for the treatment of arginine-auxotrophic tumors, such as hepatocellular carcinomas and melanomas (Yoon *et al.*, 2012; Changou *et al.*, 2014; Li *et al.*, 2016; Sharma *et al.*, 2017). Mechanistically, the anticancer effects of ADI is based on the fact that arginine auxotrophic tumour cells more specifically hepatocarcinomas, melanomas, pancreatic carcinomas and few types of

leukemia have shown lack expression of argininosuccinate synthetase due to which they are unable to synthesize their own arginine. However, for rapid malignant growth they require massive amount of arginine. To fulfil their nutritional requirement, they use arginine of circulating system. The clinical administration of ADI, hydrolyzes L-arginine of circulating system into L-citrulline, and ammonia (Wang and Li, 2014) resulting in nutritional starvation which leads selective apoptosis in cancer cells (El-Sayed *et al.,* 2015). While, normal cells remain unaffected or less affected due to endogenous biosynthesis of arginine (Kim *et al.,* 2009).

The Food and Drug Administration (FDA), USA, and European Agency for the Evaluation of Medicinal Products (EMEA) have recognized PEG-ylated form of *Mycoplasma* ADI (ADI-PEG-20) for the treatment of hepatocellular carcinomas and malignant melanomas.

Beside *Mycoplasma* ADI, many scientists have reported ADI from various microbiological sources viz. *Halobacterium salinarium* (Monstadt *et al.,* 1990) *Giardia lamblia* (Li *et al.,* 2009) *Porphyromonas gingivalis* (Rodríguez *et al.,* 2009), *Pseudomonas aeruginosa* (Oudjama *et al.,* 2002; Kundu *et al.,* 2009), *Lactococcus lactis* (Kim *et al.,* 2009) *Pseudomonas plecoglossicida* (Ni *et al.,* 2011), *Lactobacillus sakei* (Rimaux *et al.,* 2012). *Streptococcus pyogenes* M49 (Hering *et al.,* 2013), *Aspergillus fumigatus* KJ434941 and (El-Sayed *et al.,* 2015) *Enterococcus faecium* GR7 (Kaur and Kaur, 2016) but still *Mycoplasma* ADI have used in clinics. However, the curative effect of *Mycoplasma* ADI is associated with serious cytotoxicities (Fiedler *et al.,* 2015). Additionally, short serum half-life and low proteolytic tolerance are few other drawbacks of currently available ADI. As we know that ADI is considered as a strong antineoplastic agent and widely used against melanoma, hepatocarcinoma and some leukemia due to which the day by day demand of enzyme is continuously increasing. For the fulfilling of this demand, some scientist have tried to cloned and over-expressed *Mycoplasma*, *Lactococcus* and *Pseudomonas* ADI in *E. coli* but they got only limited success (Takaku *et al.,* 1995; Kim *et al.,* 2007) and the total cost of production is relatively high due to application of expensive chemicals and buffers (Kaur and Kaur, 2016). Therefore, low productivity is also one of the major limiting factors which restricted its clinical applications. Growing biotechnological advancement suggests that each organism has their own nutritional and environmental requirements; therefore no defined medium has established for the optimum production of ADIs from different microbial species (Chidambaram *et al.,* 2009; Sharma *et al.,* 2015). Hence, screening and evaluation of environmental and nutritional requirements for microorganisms are an important step for the enhanced productivity and all over economic bioprocess development. Our group is working on anticancer enzymes of microbial origin (Sharma *et al.,* 2014; Sharma and Husain, 2015; Husain *et al.,* 2016a and 2016b; Husain *et al.,* 2017). In our previous endeavour, to achieve most potent ADI producer, we isolate more than hundred indigenous bacterial strains from various environments and screened them for ADI activity. To achieve most potent ADI, the crude enzymes of these strains were further screened for *in vitro* serum half-life, proteolytic tolerance against trypsin and proteinase-K and anticancer activity. Based on them, bacterial strain PS2, isolated from rhizosphere of *Pisum sativum* recorded as potent and effective ADI producer characterized as *P. aeruginosa* PS2 (Sharma *et al.,* 2017). In the present study, we attempt

to optimize production medium using one factor at a time approach for the enhanced production of ADI obtained from *P. aeruginosa* PS2.

Materials and Methods

Anhydrous L-arginine, L-asparagine, L-glutamine, sucrose, maltose, starch, galactose, lactose, melibiose, glucose, xylose, pyruvate, gelatin, tryptone, beef extract, ammonium oxalate, potassium nitrate, casein, ammonium chloride, urea, yeast extract, NaCl, $CaCl_2$, K_2HPO_4, $MgSO_4$, KCl, trichloroacetic acid (TCA), Thiosemicarbazide (TSC), Diacetylmonoxime (DAMO), H_2SO_4, H_3PO_4 and Folin-Ciocalteu's phenol reagent were purchased from Himedia, Mumbai, India. All other chemicals were used of analytical grade and purchased from standard sources.

Bacterial strain and growth condition

ADI producing bacterium *P. aeruginosa* PS2 was obtained from Bacterial Germplasm Collection Centre (BGCC no: 2411), Rani Durgavati University, Jabalpur (M.P.), India, which was previously isolated in our Lab from rhizosphere of *Pisum sativum*. The 16S rRNA gene sequence of the strain has been deposited in NCBI Genbank data base with the accession number KF607097 (Sharma *et al.,* 2017). The strain was maintained on Luria-Bertani (LB) agar slant (pH 7) and stored at 4 °C. Stock culture was transferred to fresh LB agar slant after every 4 weeks. M-9 broth medium containing (L^{-1}): 6g $Na_2HPO_4.2H_2O$, 3g KH_2PO_4, 0.5g NaCl, 5g L-arginine, 2ml 1M $MgSO_4.7H_2O$, 1ml 0.1M $CaCl_2.2H_2O$, and 2g glucose (pH 7), was used for optimization study.

Determination of the L-arginine deaminase assay

ADI activity was quantified by measuring the formation of L-citrulline from L-arginine by following the method of Liu *et al.,* (1995). The reaction mixture containing 100 µl enzyme preparation and 900 µl of pre-warmed 0.01 M L-arginine prepared in 0.05 M phosphate buffer (pH 7). The contents of tube was mixed by vortexing and incubated for 30 min at 37 °C. Subsequently, 100 µl of 1.5 M trichloroacetic acid (TCA) was added to terminate enzyme reaction and centrifuged at 10,000 rpm for 5 min at 22 °C. Further, 1 ml of acid mixture (H_3PO_4-H_2SO_4, 3:1 v/v) was added in tube containing 500 µl supernatant and 250 µl of 1.5% diacetylmonoxime (dissolved in 10% methanol). The content of tube was vortexed and incubated at 100 °C for 15 min. The absorbance A_{530} values were measured against the control prepared by addition of TCA before enzyme addition. The amount of citrulline produced in the reaction was calculated on the basis of standard curve prepared with L-citrulline. One unit of ADI activity is defined as the amount of enzyme catalyzing 1 µM of L-arginine into 1 µM of L-citrulline per min under standard assay conditions. Specific activity of ADI is expressed as unit mg^{-1} protein. Total protein concentration was determined by the method of Lowry *et al.,* (1951), using bovine serum albumin (BSA) as the standard.

Optimization of process parameters for ADI production under shake flask culture

The ADI production by *P. aeruginosa* was optimized under shake flask culture. The effect of different physical fermentation process parameters i.e. batch time, inoculum age, inoculum size, initial pH, and incubation temperature on ADI production were studied. Effect of various nutritional parameters viz. carbon sources, nitrogen sources, salts and inducers (amino acids) on ADI production from *P. aeruginosa* PS2 were investigated separately, by varying one factor at a time method and by keeping other factor constant. At every step, the factors of previous

experiments were selected and incorporated in the semi-basal medium that increased ADI activity obtained from *P. aeruginosa* PS2.

Primary inoculum preparation effect of batch time

For inoculum preparation, a loopfull of logarithmic phase (24 h) pure culture of *P. aeruginosa* PS2 was transferred in 20 ml of aforementioned sterile medium. The flask was incubated overnight at 37°C in a rotary shaking incubator at 180 rpm. In order to determine batch time (5, 10, 15, 20, 25, 30, 35 and 40 h), 2% (v/v) inoculum (A600 = 0.6-0.8) was inoculated in 100 ml of semisynthetic broth medium and flask was incubated at 37 ºC with shaking at 180 rpm. After 5 h of intervals, 5 ml medium was withdrawn, centrifuged at 10000 rpm at 4 °C and supernatant was used to investigate ADI activity by standard ADI assay.

Effect of age of inoculum and size of inoculum

In order to determine the effect of inoculum age and inoculum size (%) on ADI production, inoculum of different ages (5, 10, 15, 20, 25, 30 and 35 h) and different sizes (1, 2, 3, 4, 4, 5, 6, 7, 8, 9, 10, and 12% v/v) was used to inoculate in 250 ml flask containing 30 ml minimal medium. Flasks were incubated at optimized incubation period at 37 ºC with shaking at 180 rpm for optimized batch time (25 h). After incubation culture was centrifuged and supernatant was used as crude enzyme. The ADI activity was analysed by standard ADI assay.

Effect of pH and temperature

The effect of different pH (4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, and 9) and different temperature (20, 25, 30, 35, 40, and 45 °C) on ADI activity was investigated. The medium with pH 7.0 and temperature 37 °C were set as a control. The flask containing 30 ml minimal medium was incubated at above mentioned optimized conditions at 37 ºC with shaking at 180 rpm for optimized batch time (25 h). Culture was harvested by centrifugation at 10000 rpm (4 ºC) for 5 min and supernatant was used as crude enzyme. The ADI activity of the crude enzyme was measured by using standard ADI assay.

Effect of carbon and nitrogen sources

To determine the influence of different carbon sources on ADI activity, various carbon sources (0.5%) such as sucrose, maltose, glucose, starch, galactose, lactose, melibiose, xylose and pyruvate were substituted in the medium. Then, to study the effect of different nitrogen sources on ADI activity various alternative of nitrogen compounds (0.3%) such as peptone, gelatin, tryptone, beef extract, ammonium oxalate, potassium nitrate, casein, ammonium chloride, urea and yeast extract were substituted in the medium and above mentioned optimized parameters were remain constant. The minimal medium containing flasks were incubated at 37 ºC with shaking at 180 rpm for optimized batch time (25 h). After appropriate incubation, culture was harvested by centrifugation at 10000 rpm for 5 min at 4 ºC and ADI activity was analyzed by standard ADI assay.

Effect of mineral ions and amino acids

The effect of various ions sources such as NaCl, $CaCl_2$, KH_2PO_4, K_2HPO_4, $MgSO_4$ and KCl (0.3%) were used in 30 ml medium containing flask, individually. Flasks were incubated at above mentioned optimized process parameters at 180 rpm for 25 h. ADI activity was analysed by standard ADI assay. Further, to study the effect of different amino acids on ADI activity, various amino acids (1%) were substituted individually in flask

containing 30 ml medium. Flasks were inoculated and incubated at previous mentioned optimized conditions. ADI activity was analysed by standard ADI assay.

Statistical analysis

All experiments were performed in triplicates and data reported as mean ± SD. Statistical analysis was done by using student t-test and p value < 0.05 was considered to be statistically significant in this study.

Results and Discussion

Effect of batch time, inoculum age and inoculum size on ADI production from *P. aeruginosa* PS2

Physical parameters including batch time, inoculum age, inoculum size, pH, temperature, and aeration greatly influence the production of microorganisms based products like enzymes, vitamins, amino acids, various alcohols and acids. Hence, in the present study, we optimized various physical parameters for maximum yield of ADI from *P. aeruginosa* PS2. As we know that batch time play a very substantial role in economic bioprocess development. Therefore, in order to determine appropriate batch time for maximum ADI yield culture flask was incubated at 37 °C (180 rpm) and after every 5 h, ADI activity was analyzed. As the results represented in Figure 1a, showed that maximum ADI activity 3.32±0.17 IU ml^{-1} was observed after 25 h of incubation. In the contrary, maximum growth (1.5±0.08) was achieved at 30 h of incubation. For the maximum yield of ADI, optimization studies were conducted with various ages of inoculum. According to the results that are represented in Figure 1b, maximum ADI yield 4.41±0.16 was achieved with 20 h old inoculum. However, ADI activity was decreased at above and below 20 h age of inoculum was used. As mentioned above, physical parameters including size of inoculum (%) also had a significant influence on ADI yield. Hence for maximum yield of ADI, we had also optimized the inoculum size. According to the results that are summarized in Figure 1c, maximum ADI yield (6.19±0.23 IU ml^{-1}) was obtained with 8% inoculum of 20h old.

Effect of pH and temperature on ADI production from *P. aeruginosa* PS2

The pH and temperature are considered as critical parameters and plays very significant role in biosynthesis of microbial origin products. Hence, to determine suitable pH for maximum yield of ADI from *P. aeruginosa* PS2 culture medium with different pH, optimized inoculum was incubated at 37 °C and 180 rpm in rotatory incubator for optimized incubation time (25 h). After incubation culture were harvested and ADI activity was investigated. According to the results presented in Figure 1d, pH of 6.5 was found optimal for ADI yield (7.92±0.32 IU ml^{-1}). Indeed, further increase in pH the yield of ADI was decreased in pH dependent manner. However, maximum growth of *P. aeruginosa* PS2 was found at pH 7.0, indicating that pH 7.0 was suitable for luxuriant growth of *P. aeruginosa* PS2 but pH 6.5 favours maximum biosynthesis of ADI. The maximum biosynthesis of ADI (8.73±0.41 IU ml^{-1}) from *P. aeruginosa* PS2 was achieved at 37 °C (Figure 1e). However, above and below this temperature (37 °C) enzyme activity was decreased in temperature dependent manner.

Effect of carbon and nitrogen sources

The nutritional requirement of each organism is different and varies according to the phase of growth and physical environment at which they are grown. Sometimes, same microbial

species isolated from different environment required different nutritional components for proper growth and development. Therefore, nutritional parameters always play a very significant role in economic bioprocess development. In the present investigation, to achieved maximum biosynthesis of ADI from *P. aeruginosa* PS2, various sugars including monosaccharides, disaccharides and polysaccharides were tested. As the results are presented in Figure 2a, the maximum ADI was 10.39±0.34 IU ml^{-1}, recorded with galactose, indicated that galactose is the best carbon source for ADI biosynthesis from *P. aeruginosa* PS2. In other tested sugars, followed by galactose milk sugar lactose (8.23±0.42 IU ml^{-1}) was significantly enhanced the production of ADI. However, monosaccharide sugar xylose did not affect the production of ADI. Further, different nitrogen sources like gelatin, tryptone, beef extract, ammonium oxalate, potassium nitrate, casein, ammonium chloride, urea, peptone, and yeast extract were amended in the production medium to determine their impact on ADI production from *P. aeruginosa* PS2. As the results are summarized in Figure 2b, peptone as nitrogen sources enhanced (13.34±0.54 IU ml^{-1}) the ADI yield followed by yeast extract (12.03±0.62 IU ml^{-1}).

Effect of mineral ions and amino acids

Mineral ions and amino acids required by the cell primarily for the synthesis of nucleic acids, phospholipids and proteins. Hence, in order to select the most favourable mineral ion source and amino acid for enhancing the production of ADI from *P. aeruginosa* PS2, experiments were performed with various mineral ions and amino acids. According to the results that are summarized in Figure 2c, we observed that KH_2PO_4 is the best mineral ion source for ADI production (16.09±0.63 IU ml^{-1}). Amino acids act as the inducers for biosynthesis of enzyme. Therefore, in the present investigation various amino acids were individually incorporated in production medium and noticed that ADI was essentially required by *P. aeruginosa* PS2 for maximal biosynthesis of ADI.

As depicted from the Figure 2d, the highest yield of ADI (17.01±0.72 IU ml^{-1}) was achieved by addition of L-arginine in minimal medium. The results of our amino acid incorporation suggest that amino acid is more significant for ADI production.

Since the discovery of enzyme to date, several enzymes were established as potent therapeutic agents. Among them ADI is one of the most important and best characterized enzymic drugs. The earlier application of ADI is focused against the treatment of hepatocellular carcinomas but in the recent past, scientific community trying to search new therapeutic applications of ADI in treatment of other arginine auxotrophic tumors such as pancreatic cancer (Liu *et al.*, 2014), prostate cancer (Changou *et al.*, 2014), leukemia (Miraki-Moud *et al.*, 2015), colon cancer (El-Sayed *et al.*, 2015), and breast cancer (Li *et al.*, 2016). As we know that, ADI is a significant player of ADI or arginine dihydrolase (ADH) pathway and generating one molecule of ATP by phosphorylation of ADP. Hence, the occurrence of ADI was reported in various groups of organisms including archaea, eubacteria and eukarya but for therapeutic applications microorganisms especially bacteria have proven to be very efficient and inexpensive sources of this enzyme. Because each organism has its own nutritional requirement therefore, screening and evaluation of the environmental and nutritional requirements of microorganisms are important steps for over all bioprocess development. In the present investigation, various physical, environmental and nutritional parameters were optimized for maximum yield of ADI from *P. aeruginosa* PS2.

Fig.1 Effects of various physical parameters on ADI production from *P. aeruginosa* PS2. (a) Effect of batch time, (b) effect of age of inoculum, (c) effect of inoculum size, (d) effect of medium pH and (e) effect of incubation temperature. After completion of each parameter ADI activity was analyzed by standard ADI assay and growth was measured by taking optical density at 600 nm. All experiments were performed in triplicate and error bar represents the ± SD of three experiments

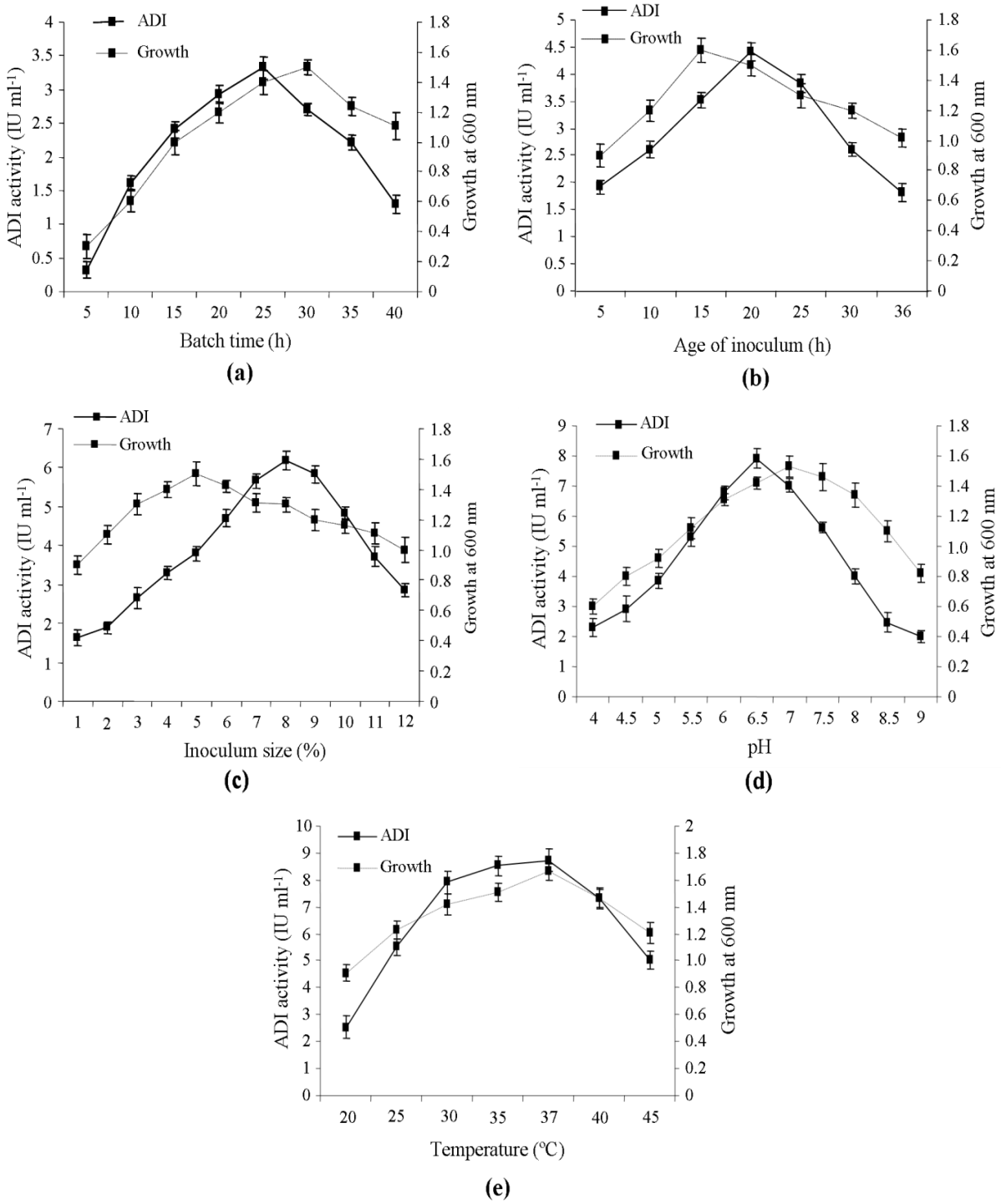
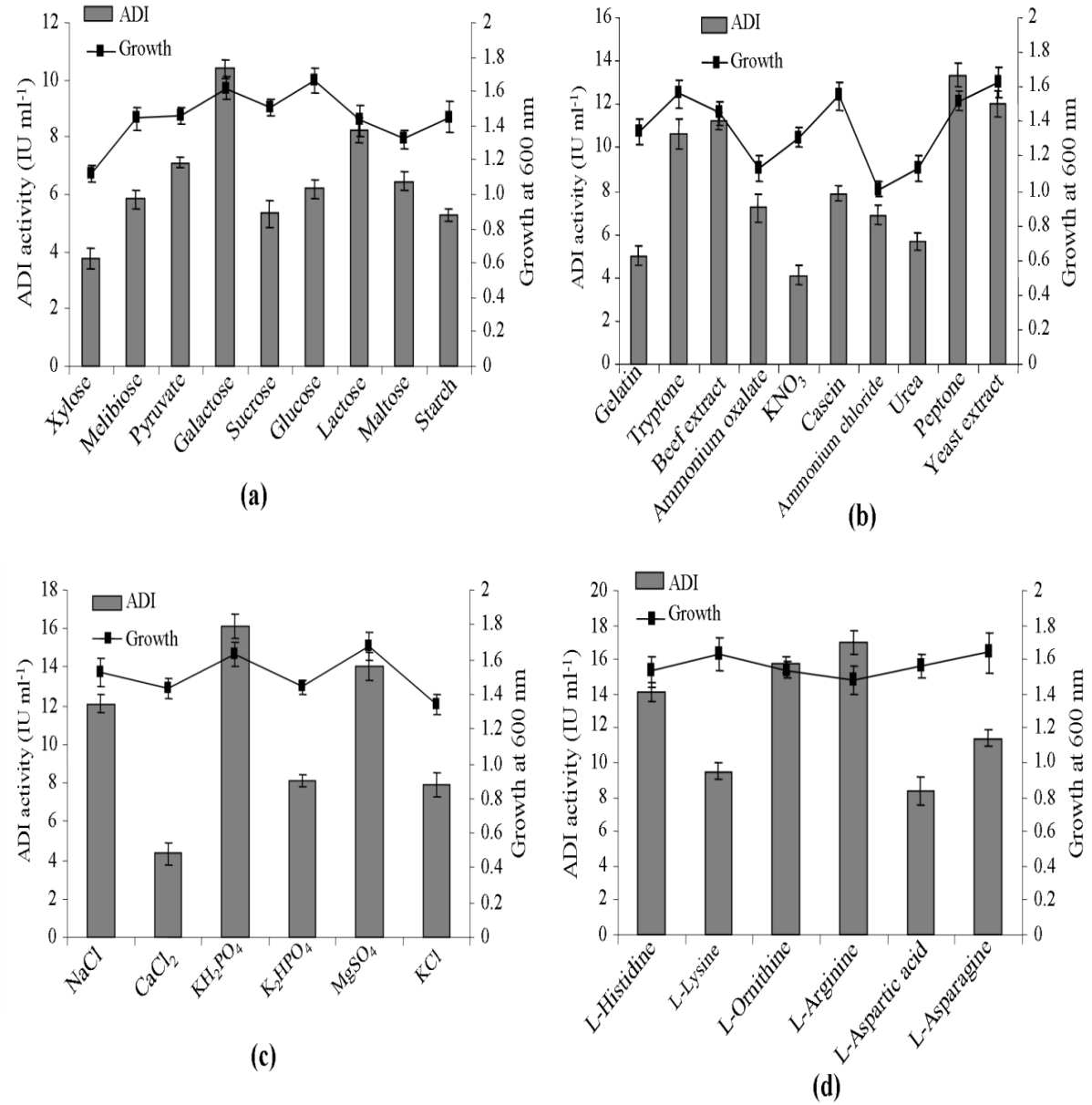


Fig.2 Effect of various nutitional parameters on ADI production from *P. aeruginosa* PS2. (a) Effect of various carbon sources, (b) effect of different nitrogen sources, (c) effect of different mineral ions and (d) effect of different amino acids. ADI activity was measured by standard ADI assay as described in materials method section and growth was measured by taking optical density at 600 nm. All experiments were performed in triplicate and error bar represents the ± SD of three experiments



Our results of batch time profile showed that maximum ADI yield was achieved after 25 h of incubation while, below 15 and above 35 h of incubation ADI production was very less. Here, we suggest that this short batch time could be more significant for large scale production of enzyme.

The age of inoculum and size of inoculum (%) directly influence the production of the

enzyme because acute percent of exponentially growing inoculum reduced lag phase and exponential growth beings immediately. In our investigation, we reported that 20 h of inoculum age and 8% of inoculum size were more suitable and enhanced 1.32 and 1.86 fold of ADI production from *P. aeruginosa* PS2. Generally, in the enriched medium the generation time of *Pseudomonas* sp. is 25-35 min (LaBauve and Wargo, 2012). However, this time could be reduced or expanded which depends on genetic potential of the organism or culture condition. The 20 h old inoculum in production medium takes less time in acclimatization and reduced log phase due to which inoculated cells directly start dividing in medium and produced ADI. On the other hand, inoculum higher than 8% could be attributed to decrease in the concentration of the medium component. Our findings are in against with the previous findings of Kaur and Kaur (2013) and Liu *et al.,* (2008), they have reported that maximum yield of ADI by using 2% of inoculum from *Enterococcus faecium* GR7, *Pseudomonas plecoglossicida* CGMCC2039, respectively.

The effect of pH on ADI production was examined at various pH values ranging from 4-9. At pH 6.5, the yield of ADI was increased to 3.38 fold. Here, author suggest that the two possibilities of maximum ADI synthesis; 1) Most of the *Pseudomonas* species including *P. aeruginosa* are grown rapidly in slight acidic pH or at near to neutral pH, 2) At 6.5 pH, *P. aeruginosa* PS2 ADI might be more stable but it requires further investigations. Our observations are approximately similar to the findings of Kaur and Kaur (2013), who reported that *Enterococcus faecium* sp. GR7 produced maximum ADI at 6.0 pH. The optimum temperature for ADI production from *P. aeruginosa* PS2 was found to be at 37 °C, which increased ADI yield upto 2.6 fold. However, below 30 °C and above 40 °C, the biosynthesis of ADI activity decreased abruptly.

Carbon sources are very important nutritional constituent and required as source of energy (Sharma *et al.,* 2015). Previous communication suggest that the production of ADI is highly depend on carbon sources (Yu *et al.,* 2010; Kaur and Kaur, 2013; Patil *et al.,* 2016). In our present investigation, we observed that galactose was the best carbon source for ADI biosynthesis which enhanced upto 3.12 fold yield, which are in accordance with the previous findings (Kaur and Kaur, 2013; Crow and Thomas, 1982). However, in the presence of glucose enzyme yield was approximately half as compared to galactose. This may be happened due to catabolic repressor nature of glucose. ADI production from *P. aeruginosa* PS2 varied with various compound tested as nitrogen sources. Among them, culture medium supplemented with peptone favored maximum ADI synthesis (4.02 fold) followed by yeast extract (3.62 fold). To select the most favorable mineral ion and amino acid for enhanced production of ADI, experiments were performed using various mineral ion sources and amino acids individually. The presence of K^+ (potassium) ions in the medium improved the enzyme productivity approximately upto 4.8 fold. However, in the presence of arginine in the medium improved the enzyme productivity approximately to 5.12 fold. Our observation are in consistent with recent observations of Yu *et al.,* (2010), Kaur and Kaur, (2013), Patil *et al.,* (2016) who suggested that arginine act as inducer for ADI production but more in-depth studies are needed for strengthening our current findings.

In conclusion, present study demonstrated the optimization of cultural and nutritional conditions for production of ADI from indigenous bacterium *P. aeruginosa* PS2. The

maximum biosynthesis of ADI from this strain was achieved when batch time 25 h, inoculum age of 20 h, inoculum size 8%, initial pH 6.5 and incubation temperature 37 °C were applied. However, galactose, peptone, potassium dihydrogen phosphate and arginine had affirmative effect as best carbon, nitrogen, mineral ions and inducer sources, respectively. The results of present study suggested that *P. aeruginosa* PS2 could be used for large-scale production of ADI but further studies are required to validate the current findings.

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