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Enhancing Production of Amino Acids from *Bacillus* spp. Using Batch and Fed-batch Fermentation Strategies

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Author's contributions

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

Aims: Production of amino acids from black strap sugar cane molasses by *Bacillus* sp. R22EG1 strain and *Bacillus* sp. R20EG2 using batch and fed-batch (pulsed and continuous feeding) cultures was investigated to achieve the maximum concentration of free amino acids.
Place and Duration of Study: Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, Cairo, Egypt, between December 2011 and March 2012.
Methodology: Two *Bacillus* strains namely R22EG1 and R20EG2, were used as amino acid produces. The amino acid producing bacteria were grown in the bioreactor with batch and fed-batch cultivation. The fed-batch fermentations were performed in two strategies using pulsed and continuous feeding of black strap sugar cane molasses. In the first strategy (fed-batch by pulsed feeding), the amount of black strap sugar cane molasses (50 $ml.L^{-1}$) was added to the fermentation vessel. Two, three and four additions of this amount of black strap sugar cane molasses were also added during the first 12 and 48 h of cultivation periods**.** In the second strategy, the black strap sugar cane molasses was fed continuously during the first 12, 18 and 24 h of cultivation periods at rates of 4.17, 2.78 and 2.08 $ml.L^{-1}.h^{-1}$, respectively (fed-batch by continuous feeding). The cell dry weight, amino acid concentration and residual sugar were determined as well as the growth and production parameters were calculated.
Results: The biological activity of *Bacillus* sp. R22EG1 and *Bacillus* sp. R20EG2 strains during

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production of amino acids on 5% black strap sugar cane molasses medium for 72 h at 30℃ was investigated in bioreactor as a batch and fed-batch cultures. In batch culture, the highest figures of amino acid concentration (2.30 and 2.83 $g.L^{-1}$), yield (9.52 and 11.71%), and conversion coefficient (11.09 and 13.44%) were recorded after 72 h and 60 h of fermentation periods by *Bacillus* sp. R22EG1 and R20EG2 strains, respectively, whereas the maximum productivity, approximately, 0.044 and 0.059 $g.L^{-1}.h^{-1}$ were observed after 12 h and 24 -36 h for *Bacillus* sp. R22EG1 a nd R20EG2 strains, respectively. Two feeding strategies (pulsed and continuous) were studied during production of amino acids using fed-batch culture. The highest cell dry weight, amino acid concentration and yield were recorded after three pulsed molasses addition during the first 12 h of fermentation periods with specific addition rate of 0.204 $ml.L^{-1}.h^{-1}$ (0.099 $g.L^{-1}.h^{-1}$ sugar) for the tested strains. The continuous feeding rate of 4.17 $ml.L^{-1}.h^{-1}$ (2.01 $g.L^{-1}.h^{-1}$ sugar) was more favorable than pulsed feeding during 12 h for amino acid production in fed-batch culture, as it increased the amino acid concentration by *Bacillus* sp. R22EG1 and R20EG2 strains, approximately, (1.53 & 1.42) fold than pulsed feeding and about (1.64 & 1.59) fold than that produced in batch bioreactor technique, after a 48 h fermentation period. The highest content of free amino acid species in culture supernatants was glutamic acid produced by both strains.

Conclusion: The maximum production of amino acids from continuous fed–batch by *Bacillus* sp. R22EG1 strain and *Bacillus* sp. R20EG2 was 3.76 and 4.49 $g.L^{-1}$ with continuous feeding at 4.17 $ml.L^{-1}.h^{-1}$ (2.01 $g.L^{-1}.h^{-1}$ sugar) at 48 h, respectively. These results were 1.53 & 1.42 fold higher than pulsed feeding and 1.64 & 1.59 fold higher than batch fermentation by *Bacillus* sp. R22EG1 and R20EG2 strains, respectively. The highest content species of free amino acids was glutamic acid using a Bichrom 30 amino acid analyzer.

Keywords: Amino acids; bacteria; pulsed fed–batch; continuous fed–batch; bioreactor.

1. INTRODUCTION

Since 1950s, the production of amino acids by fermentation has become an essential technology of Industrial microbiology. This has led to numerous studies to understand and improve the metabolic conditions driving to amino acid overproduction. Industrial fermentation processes have been developed for large scale production of amino acids. On a commercial scale, fermentation is generally conducted using aerated agitated tank fermentors or airlift tank fermentors in the 50- to 500-kl size range. With increases in the demand for amino acids and the needs for cost reduction to remain competitive, there have been a gradual increase in the size of fermentors and this trend will continue. The parameters affecting production yields in the fermentor cultivation include aeration, agitation, pressure within the tank, feeding rate of sugar, pH, and temperature. Among these, the first four parameters must be given special attention because their optimal conditions could vary considerably depending on the type of the fermentor and the scale of the operation [1]. Fermentation processes have become a common practice for overproduction of amino acids nowadays as it is cheaper and easier than other processes for commercial production of methionine and other essential amino acids [2,3,4]. Batch culture fermentation was the most important process operation used in bio-processing industries due to its simplicity but less suitable when cell growth was inhibited with by- products formation [5]. Batch technology is applied as a simple process in terms of process control (no nutrient supply necessary), technology (no vessel for feed solution necessary) or sterility (less feed lines). Major boundary for a possible intensification of a batch process is the osmolarity of the initial medium. Especially high initial concentrations of the carbon source interfere with growth and productivity of *coryneform* bacteria [6,7]. Some problems of batch culture fermentation can be overcome by using a fed batch procedure. Fed batch culture is a batch culture, which substrate nutrients are fed continuously or sequentially without the removal of fermentation broth. It is widely used for the production of microbial biomass, ethanol, organic acids, antibiotic, vitamins, enzymes, and other compound [8,9]. The important rationale of fed batch operations are the excess of carbon caused inhibition of cell growth and the excess of carbon or nitrogen caused catabolite repression [10]. The production processes should be run in the fed-batch mode, not only to increase productivity and to prevent the formation of inhibiting overflow metabolites, but also to increase process reproducibility [11,12,13]. Corynebacteria are the primary group of microorganisms used in the

production of amino acids with several strategies [14], although the other genera such as *Escherichia*, *Serratia* and *Bacillus* (Prokaryotes) and *Hansenule*, *Candida* and *Saccharomyces* (Eukaryote) also have commercial importance [15].

In the present work, production of amino acids from black strap sugar cane molasses by *Bacillus* sp. R22EG1 and *Bacillus* sp. R20EG2 strains using batch and fed-batch (pulsed and continuous feeding) cultures were investigated to achieve the maximum concentration of amino acids.

2. MATERIALS AND METHODS

2.1 Microorganisms, Media and Inoculum Preparation

Bacillus sp. R22EG1 and *Bacillus* sp. R20EG2 strains were used as producers of amino acids in this investigation. These strains were previously isolated from soil rhizosphere in Kalubia Governorate, Egypt and identified using phenotypic characteristics, phylogenetic analysis based on 16S rRNA gene sequence (data submitted to NCBI GenBank) [16]. These strains were maintained by transferring them at regular intervals on nutrient agar slants incubated at 30℃ for 24 h and stored at 4℃. To prepare the inoculum, a loopful of both strains was inoculated in 100 ml Erlenmeyer flasks containing 50 ml of sterile nutrient broth medium individually. Then the inoculated flasks were incubated at 30℃ on rotary shaker at 150 rpm for 24 h. The transfer inoculum was 1% (v/v) for the fermentation experiments. Fermentations were carried out in modified molasses medium reported by Abou-Taleb and Sayed, [16] and consisted of (g/100 ml): Black strap sugar cane molasses, 5.0 ml; $CaCO_3$, 2.0; KH_2PO_4, 0.05; K_2HPO_4, 0.05; $MgSO_4.7H_2O$, 0.025; soybean husk, 2.0 and pH adjusted to 7.2. *$CaCO_3$ was added after sterilization of medium in an autoclave for 15 min at 121℃.

2.2 Batch and Fed-Batch Fermentation in Stirred Bioreactor

The bioreactor used in this study was a 3 litre dished bottom bioreactor Z 6110 / coob (Cole – Parmer Instruments), which consisted of 3 litres vessel equipped with lipseal stirrer assembly, automatic pH controller, automatic dissolved O_2 controller, CO_2 controller, automatic temperature controller, foam controller and multi-channel peristaltic pump (for feeding). The amino acids producing bacteria were grown in the bioreactor as batch and fed-batch (pulsed and continuous) cultures. Fermentations were carried out, at a controlled temperature of 30℃, dissolved O_2 level at 20% of air saturation and 500 rpm agitation speed. Initial pH was adjusted to 7.0±0.1 which was not controlled during the fermentation period. Samples of 10 ml were taken periodically. Each sample was centrifuged at 10,000 rpm/10 min at 4℃ and the pellet was collected to determined cell dry weight. The supernatant was used to determine concentration of amino acids and reducing sugar.

2.2.1 Batch fermentation procedure

The fermentor containing 1980 ml of productive medium was firstly sterilized at 121℃ for 15 min. Twenty milliliters of inoculum were added aseptically into the fermentor. Samples of 10 ml were withdrawn from the fermentation broth at regular time intervals. The final working volume was 2 litres.

2.2.2 Fed-batch fermentation procedure

The fermentation was performed in a 3 litres stirred fermentor with effective working volume of 1 litre at the end of feeding period. The fermentations were performed in two strategies using pulsed and continuous feeding of black strap sugar cane molasses. In the first strategy (fed-batch by pulsed feeding), the amount of black strap sugar cane molasses (5 ml/ 100ml medium) was added to the fermentation vessel. Pulsed addition of black strap sugar cane molasses was carried out every 12 or 24 h during the first 48 h of fermentation periods. Two, three and four pulsed additions were applied during the first 12, 12 and 36 h of fermentation periods with a specific addition rate of 0.116 $ml.L^{-1}.h^{-1}$, 0.204 $ml.L^{-1}.h^{-1}$ and 0.091 $ml.L^{-1}.h^{-1}$, respectively. In the second strategy, the black strap sugar cane molasses was fed continuously at a rate of 4.17, 2.78 and 2.08 $ml.L^{-1}.h^{-1}$ during the first 12, 12 and 24 h of fermentation periods, respectively (fed-batch by continuous feeding).

2.3 Analytical Procedures

The pellet (biomass) was washed twice with distilled water, then dried at 80℃ to constant weight. The total amount of free amino acids (quantitative analysis) was determined in the supernatant using acidic ninhydrin method

(colorimetric method) as described by Chinard [17], (16 ml of 0.6 M phosphoric acid was mixed with 64 ml of glacial acetic acid and 1 g of ninhydrin to prepare 80 ml of ninhydrin reagent) ninhydrin most free amino acids are oxidatively deaminated to form ammonia, and ninhydrin is reduced to hydrindantin. Ammonia and hydrindantin react to form the purple product diketohydrindylidene diketohydrindamine (purple) when heated that can be quantified at 570 nm. The amino acid concentration in the sample was determined by referencing to a standard curve of known concentration for glutamic acid. Identification of free amino acids produced by tested strains using amino acid analyzer (Bichrom 30) according to Horwith [18] AOAC Method 982.30, sample equal to 10 mg of protein was weighted in the conical flask and 25 ml 6N HCL was added to the sample. The flask was placed in an oven at 110℃ for 24 h. Then was opened and rotary evaporator was used to reduce the volume to 5- 10 ml under vacuum at 40-50℃. Suitable volume of sodium citrate buffer (pH 2.2) was added to hydrolyzed sample. After all soluble material completely dissolved, the sample is ready for analysis. Residual sugar was determined in the supernatant using potassium ferricyanide method [19], one ml sample and add 1.5 ml of alkaline potassium ferricyanide solution, mixed well, and boiled in water bath for exactly, 20 min, cooled, and then the volume was raised up to 10 ml with distilled water. Optical density was taken against isopropyl solution at 420 nm. The specific rates: growth rate (μ_x), production of amino acids (μ_p) and sugar consumption (μ_s) were calculated according to Doelle [20]. Amino acid yield (%), conversion coefficient (%), effective yield (%) and sugar utilization efficiency (%) were calculated according to Ramadan et al. [21]. Amino acid productivity ($g.L^{-1}.h^{-1}$) and amino acid yield coefficient relative to biomass ($Y_{A/x}$) were calculated according to Lee [22] and Grothe et al. [23], respectively.

2.4 Statistical Analysis

The correlation coefficient between samples was analyzed with Microsoft Office Excel (2010).

3. RESULTS AND DISCUSSION

3.1 Batch Culture

From our previous investigation (Abou-Taleb and Sayed [16]), we concluded that the highest production of amino acids could be obtained by *Bacillus* sp. R22EG1 and R20EG2 strains grown on black strap sugar cane molasses supplemented by soy bean husk and the nutritional elements of the basal medium using shaking flasks as a batch culture for 72 h at 30℃. Therefore, it was found valuable to optimize the environmental factors for production of amino acids by *Bacillus* sp. R22EG1 and R20EG2 strains using the above medium in a bioreactor as a batch culture. Here in this study, the highest level of cell dry weight achieved on 72 h by *Bacillus* sp. R22EG1 strain was 1.99 $g.L^{-1}$ with sugar utilization efficiency of 85.88%, consumed sugar of 20.74 $g.L^{-1}$, amino acid yield of 9.49%, effective yield of 3.98%, conversion coefficient of 11.09% and the highest concentration of amino acids being 2.30 $g.L^{-1}$ (Fig. 1a). Whereas, the highest figures of amino acid yield coefficient relative to biomass ($Y_{A/x}$) 1.16 $g.g^{-1}$ and amino acid productivity of 0.044 $g.L^{-1}.h^{-1}$ were recorded after a 12 h fermentation period. With respect to specific growth rate (μ_x) of 0.032 h^{-1} with a positive correlation coefficient (R^2= 0.927), specific production of amino acids rate (μ_p) of 0.024 h^{-1} with a positive correlation coefficient (R^2= 0.918) and specific sugar consumption rate (μ_s) of 0.017 h^{-1} with a positive correlation coefficient (R^2= 0.975) were calculated during 6-60 h, 12-72 h and 24 - 48 h of fermentation periods, respectively. However, the highest figure of amino acid production by *Bacillus sp.* R20EG2 strain was found to be on the 60 h of propagation being 2.83 $g.L^{-1}$ with cell dry weight 2.00 $g.L^{-1}$, sugar utilization efficiency of 87.12% consumed sugar of 21.04 gl^{-1}, amino acid yield of 11.71%, effective yield of 4.00% and conversion coefficient of 13.44%. Whereas, the highest figures of amino acid yield coefficient relative to biomass ($Y_{A/x}$) 1.54 g. g^{-1} was recorded after 24 h of fermentation periods. The maximum amino acid productivity of 0.059 $g.L^{-1}.h^{-1}$ was recorded during 24 -36 h of the fermentation period (Fig. 1b). Also, the specific growth rate (μ_x) of 0.028 h^{-1} with R^2 value (0.956), specific amino acid production rate (μ_p) of 0.030 h^{-1} with R^2 value (0.887) and specific sugar consumption rate (μ_s) of 0.031 h^{-1} with R^2 value (0.816) were calculated during 12 - 48 h of fermentation periods, respectively. The data were analyzed statistically and a high positive correlation coefficient (R^2) ranged between 0.89 - 0.98 were detected between incubation periods and each of cell dry weight, amino acid concentration, consumed sugar, sugar utilization coefficient, amino acid yield and effective yield for *Bacillus* sp. R22EG1 and R20EG2 strains. It was also interesting to notice that the positive correlation coefficient between incubation period

and amino acid yield coefficient relative to biomass ($Y_{A/x}$) was less than the previous values being 0.52 and 0.51 for *Bacillus* sp. R22EG1 and R20EG2 strains, respectively. In batch fermentation, the concentration of amino acids was improved 1.01 and 1.14 fold as compared to shake flasks from previous study (Abou-Taleb and Sayed [16]) as a batch culture using *Bacillus* sp. R22EG1 and. R20EG2 strains, respectively. Nampoothiri and Pandey, [24] observed that production of L-glutamic acid from *Brevibacterium* sp. using cassava starch hydrolysate yielded 21 $g.L^{-1}$ glutamic acid. This production was higher approximately two and a half fold than what was obtained in a shake flask study. Lawal et al. [25] also found that L-glutamic acid yield from some strains of *Bacillus* varied among strains of *B. subtilis*, *B. licheniformis*, *B. polymyxa* and *B. pumilus* were ranged from 5 – 8.4 $mg.L^{-1}$. In addition, the production of L-phenylalanine, a metabolite concentration of 1.03 $g.L^{-1}$ *Escherichia coli* BL21 (DE3) as reported by Thanapimmetha et al. [26]. L-valine production rate of 0.08 mmol/gDCW/h obtained in the batch culture fermentation from *E. coli* strain [27].

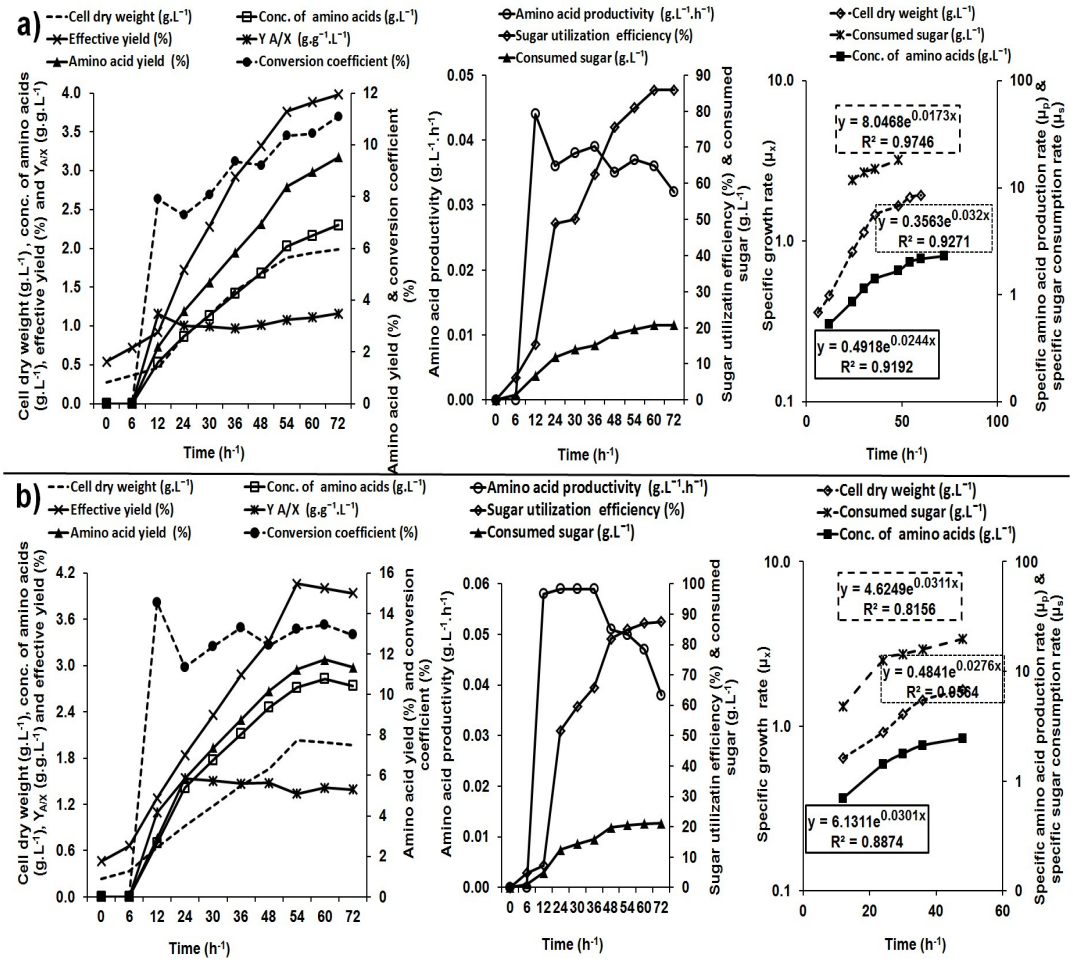

Fig. 1. Biological activity of *Bacillus* sp. strains during their propagation in bioreactor as a batch culture for 72 h at 30°C

*a) Bacillus sp. R22EG1 strain; b) Bacillus sp. R20EG2 strain; *Conc.= Concentration*

3.2 Fed-batch Culture

3.2.1 Pulsed sugar feeding

Data illustrated in (Figs. 2 and 3) reveal the impact of pulsed black strap sugar cane molasses addition on cell growth and amino acid production by *Bacillus* sp. R22EG1 and R20EG2 strains.

The highest figure of cell dry weight, amino acid concentration and amino acid yield were clearly observed at the specific addition rate of 0.204 $ml.L^{-1}.h^{-1}$ (2.11 $g.L^{-1}$, 2.45 $g.L^{-1}$ & 9.94%, respectively) after 54 h of cultivation and 0.116 $ml.L^{-1}.h^{-1}$ (1.99 $g.L^{-1}$, 2.40 $g.L^{-1}$ & 9.93%, respectively) after 60 h of cultivation by *Bacillus* sp. R22EG1 strain (Fig. 2). In contrast, the amino acid concentration and amino acid yields of 2.31 $g.L^{-1}$ and 9.55% at the specific addition rate of 0.091 $ml.L^{-1}.h^{-1}$ were obtained after 60 h of cultivations. The highest percentage of sugar utilization efficiency was 85.22%, 77.89% and 57.37 % at the specific addition rate of 0.116, 0.204 and 0.091 $ml.L^{-1}.h^{-1}$ after 60, 54 & 60 h of cultivations, respectively. The highest level of amino acid productivity was found to be on the 6^{th} hour of propagation being 0.089, 0.095 & 0.078 $g.L^{-1}.h^{-1}$ at the specific addition rate of 0.116, 0.204 & 0.091 $ml.L^{-1}.h^{-1}$, respectively. The amino acid yield coefficient relative to biomass ($Y_{A/x}$) (1.59, 1.93 & 1.77 $g.g^{-1}$) was recorded after 12 h at the specific addition rate of 0.116, 0.204 & 0.091 $ml.L^{-1}.h^{-1}$, respectively. With regard to *Bacillus* sp. R20EG2 (Fig. 3), the best specific addition rate of 0.204 $ml.L^{-1}.h^{-1}$ gave the maximal cell dry weight, amino acid concentration and amino acid yield of 2.34 $g.L^{-1}$, 3.16 $g.L^{-1}$ and 13.08%, respectively after 60 h of cultivations, followed by 0.116 $ml.L^{-1}.h^{-1}$ giving 2.13 $g.L^{-1}$ cell dry weight, 3.08 $g.L^{-1}$ amino acid concentration and 12.73% amino acid yield. On the other hand, the amino acid concentration and amino acid yield were decreased 2.98 $g.L^{-1}$ and 12.34% at the specific addition rate of 0.091 $ml.L^{-1}.h^{-1}$ for 60 h, respectively. Whereas the highest value of sugar utilization efficiency was 85.22% at the specific addition rate of 0.116 $ml.L^{-1}.h^{-1}$ followed by 0.204 $ml.L^{-1}.h^{-1}$ (82.36%), then 0.091 $ml.L^{-1}.h^{-1}$ (53.77%) after 60 h of cultivations. The productivity of amino acids was 0.135, 0.143 and 0.114 $g.L^{-1}.h^{-1}$ at the specific addition rate of 0.116, 0.204 and 0.091 $ml.L^{-1}.h^{-1}$, respectively. The amino acid yield coefficient relative to biomass ($Y_{A/x}$) (2.32$g.g^{-1}$ after 6 h, 2.01 $g.g^{-1}$ after 12 h & 1.71 $g.g^{-1}$ after 24 h) was recorded at the specific addition rate of 0.116, 0.204 & 0.091 $ml.L^{-1}.h^{-1}$, respectively. Also, the specific growth rate (μ_x), specific amino acid production rate (μ_p) and specific sugar consumption rate (μ_s) were calculated from log phase for *Bacillus* sp. R22EG1 and R20EG2 strains on productive medium using bioreactor as fed-batch culture by pulsed 5% black strap sugar cane molasses feeding at specific addition rates of 0.204, 0.116 and 0.091 $ml.L^{-1}.h^{-1}$ (Figs. 4-a & b). Results show that both strains of *Bacillus* sp. R22EG1 and R20EG2 grew exponentially during the first 6- 54 h of fermentation periods with 0.0323 h^{-1} (R^2 = 0.922) and 12-54 h of fermentation periods with 0.0278 h^{-1} (R^2 = 0.975), respectively, at the specific addition rate of 0.204 $ml.L^{-1}.h^{-1}$. The corresponding figures of specific amino acid production rate (μ_p) of 0.0314 h^{-1} with R^2 value (0.911) and specific sugar consumption rate (μ_s) of 0.0305 h^{-1} with R^2 value (0.855) were recorded at 6-48 h and 12-48 h of fermentation periods, respectively at the specific addition rate of 0.204 $ml.L^{-1}.h^{-1}$ for *Bacillus* sp. R22EG1 strain (Fig. 4 a). With regard to *Bacillus* sp. R20EG2 date illustrated in Fig. 4b showed that the highest figures of specific amino acid production rate (μ_p) (0.0234 h^{-1}) with R^2 value (0.884) and specific sugar consumption rate (μ_s) (0.0561 h^{-1}) with R^2 value (0.873) were recorded at 6-54 h and 6-36 h of fermentation periods, respectively at the specific addition rate of 0.204 $ml.L^{-1}.h^{-1}$. Hermann and Rieping [28] observed a similar peak in threonine production at 36 h of fed-batch. In Clostridia, fed batch cultures could be utilized to produce amino acid exceeding the solubility, these data correlated with our study, where significantly increased amino acid production was observed with fed-batch culturing [29].

3.2.2 Continuous sugar feeding

The biological activity of *Bacillus* sp. R22EG1 and R20EG2 strains grown on 5% black strap sugar cane molasses as fed-batch culture using continuous feeding was investigated (Figs. 5 & 6). Data clearly revealed that both cell dry weight and consumed sugar gradually increased during fermentation period at all rates of feeding. The maximum amino acid concentration was obtained after 48 h of incubation, at addition rates of feeding 4.17, 2.78 and 2.08 $ml.L^{-1}.h^{-1}$ being 3.76, 2.96 and 3.12 $g.L^{-1}$, respectively. Also here in (Fig. 5) data represented the best addition of black strap sugar cane molasses rate of was 4.17 $ml.L^{-1}.h^{-1}$which gave the highest figures of amino acid yield (15.57%), sugar utilization efficiency (95.15%), amino acid yield coefficient relative to biomass (1.59 $g.gL^{-1}$) and

amino acid productivity (0.152 $g.L^{-1}.h^{-1}$) after 48, 54, 12 and 6 h of cultivation for *Bacillus* sp. R22EG1 strain, respectively. With regard to *Bacillus* sp. R20EG2 strain results illustrated in (Fig. 6) demonstrates that the best addition molasses rate of 4.17 $ml.L^{-1}.h^{-1}$ was reached to the highest cell dry weight (3.75 $g.L^{-1}$), amino acid concentration (4.49 $g.L^{-1}$) and amino acid yield (18.57%) after 48 h of fermentation periods. In addition, we also recorded the highest figures of sugar utilization efficiency at 98.55%, amino acid yield coefficient relative to biomass at 2.04 $g.g.L^{-1}$ and amino acid productivity at 0.214 $g.L^{-1}.h^{-1}$ after 60, 12 and 6 h of cultivation, respectively. *Bacillus* sp. R22EG1 and R20EG2 strains were grown exponentially with specific growth rate (μ_x) of 0.032 h^{-1} with R^2 value (0.9190) & 0.0407 h^{-1} with R^2 value (0.9568) during the first 12-48 h and 6-48 h of fermentation period at addition black strap sugar cane molasses rate of 4.17 $ml.L^{-1}.h^{-1}$, respectively, (Figs. 7a, b). The corresponding figures of specific amino acid production rate (μ_p) (0.0168 h^{-1}) with high R^2 value (0.9397) and specific sugar consumption rate (μ_s) (0.2838 h^{-1}) with R^2 value (0.8636) were recorded at 3-12 h and 12-48 h of fermentation periods, respectively at addition black strap sugar cane molasses rate of 4.17 $ml.L^{-1}.h^{-1}$ for *Bacillus* sp. R22EG1 strain (Fig. 7a). With regard to *Bacillus* sp. R20EG2 date illustrated in (Fig. 7b), the specific amino acid production rate (μ_p) (0.0111 h^{-1}) with R^2 value (0.7622) and specific sugar consumption rate (μ_s) (0.2594 h^{-1}) with R^2 value (0.8791) were recorded at 12-48 h and 3-12 h of fermentation periods, respectively at addition molasses rate of 4.17 $ml.L^{-1}.h^{-1}$.

Furthermore sometimes the construction of new strains is necessary for repeated fed-batch processes because of increased generation numbers of the bacteria and instability of the producers as described for the production of L-phenylalanine [30]. Wild-type *B. methanolicus* MGA3 strain secreted up to 58 $g.L^{-1}$ of L-glutamate in continuous fed-batch culture reported by Schendel et al. [31], Brautaset et al. [32] and Brautaset et al. [33]. Also, L-tyrosine production using fed-batch fermentation by *E. coli* T1 and T2 was 3.8 and 9.7 $g.L^{-1}$, respectively [34]. L-valine production rate from *E. coli* strain was 0.97 mmol/gDCW/h using fed-batch fermentation [27].

From the previous data, we summarized that the continuous feeding at 4.17 $ml.L^{-1}.h^{-1}$ (2.01 $g.L^{-1}.h^{-1}$ sugar) could be more favorable than pulsed feeding for amino acid production in fed–batch culture, as it increased the amino acid concentration by both *Bacillus* sp. R22EG1 and R20EG2 strains, approximately 1.53 & 1.42 fold than pulsed feeding and about 1.64 & 1.59 fold than that produced in batch bioreactor technique, after 48 h fermentation periods. The fed-batch process could provide improved productivity by increasing yields and reducing fermentation periods. Fed batch culture was better than batch culture for the production of L-glutamic acid by *Brevibacterium* sp. (16% more the batch culture) as reported by Nampoothiri and Pandey, [24]. L-valine production rate from *E. coli* strain using fed-batch fermentation was more than 10-fold higher than the L-valine production rate obtained in the batch phase [27].

3.3 Identification of Free Amino Acids Produced by Tested Strains

The profiles of free amino acids produced in culture supernatants by *Bacillus* sp. R22EG1 and R20EG2 strains using bioreactor as fed-batch culture with continuous addition of black strap sugar cane molasses at a rate of 4.17 $ml.L^{-1}.h^{-1}$ after 48 h were represented in (Table 1). It has been recorded that only 17 out of 20 amino acids in culture supernatants of *Bacillus* sp. R22EG1 and R20EG2 strains, including the most essential amino acids (threonine, valine, isoleucine, leucine, phenylalanine, arginine, proline and cysteine) ranged from 20.60 to 3.54 mg/100ml and 32.00 to 4.74 mg/100ml for *Bacillus* sp. R22EG1 and R20EG2 strains, respectively. The non-essential amino acids (aspartic acid, serine, glutamic acid, glycine, alanine, tyrosine, arginine, proline and cysteine) were also found, ranged from 67.40 to 5.70 mg/100ml and 85.62 to 7.60 mg/100ml, respectively. The amino acids with the highest contents were identified as glutamic acid (67.40 & 85.62 mg/100 ml) followed by aspartic acid (36.70 & 48.72 mg/100 ml) for *Bacillus* sp. R22EG1 and R20EG2 strains, respectively. In *Bacillus* sp. R22EG1 strain, lysine, leucine, proline and phenylalanine were found to be in higher quantities while for *Bacillus* sp. R20EG2 strain, lysine, proline, phenylalanine, leucine, arginine and tyrosine were found to be abundant amino acid. Lawal et al. [25] also stated that the amount of L-glutamic acid was varied among *B. subtilis*, *B. licheniformis*, *B. polymyxa* and *B. pumilus* strains. *Corynebacterium glutamicum* was originally used as L-glutamate producing bacterium [33,35,36]. Also Jyothi et al. [37] and Nadeem et al. [38] reported that the maximum

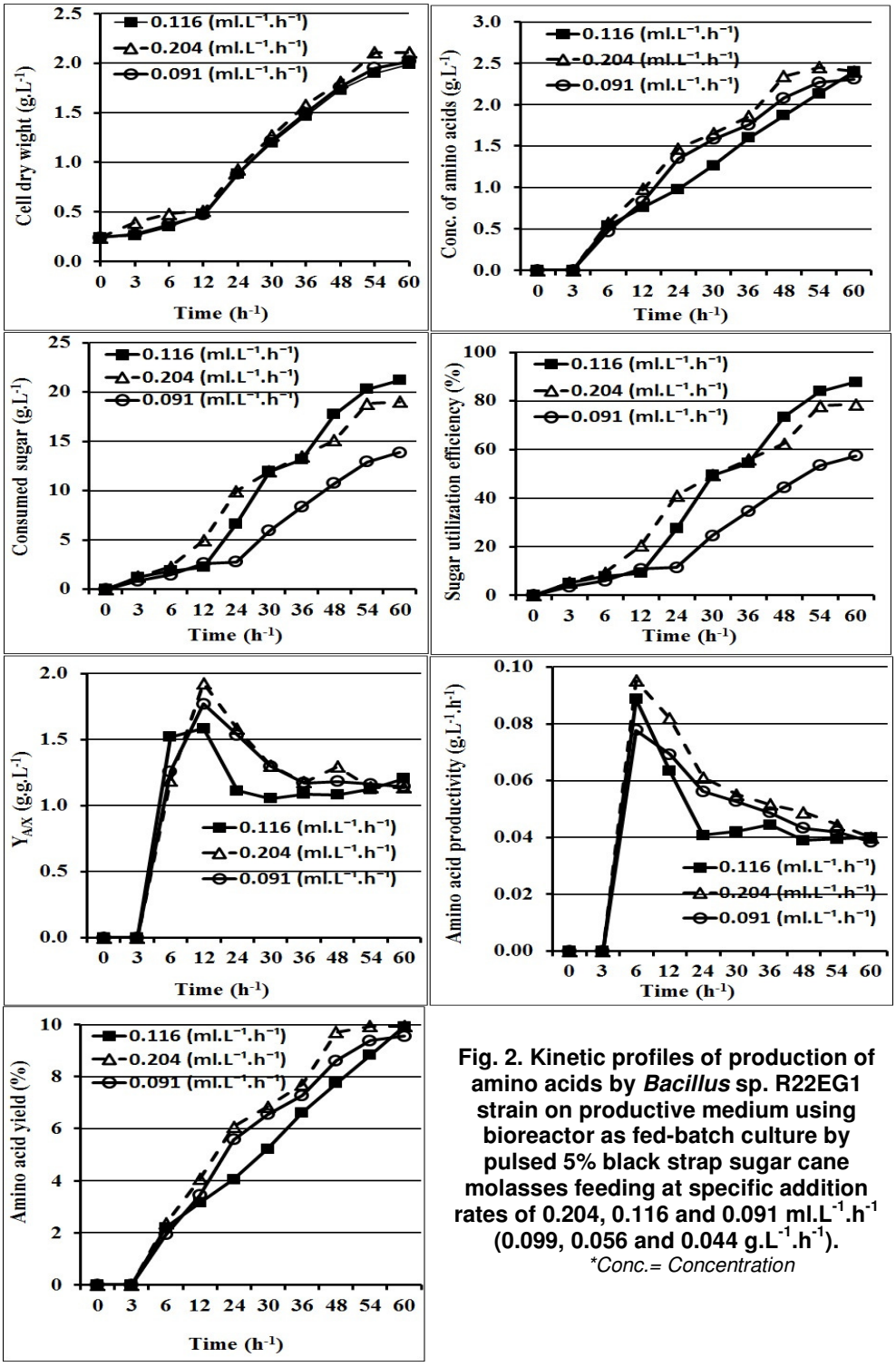

Fig. 2. Kinetic profiles of production of amino acids by *Bacillus* sp. R22EG1 strain on productive medium using bioreactor as fed-batch culture by pulsed 5% black strap sugar cane molasses feeding at specific addition rates of 0.204, 0.116 and 0.091 ml.L^{-1}.h^{-1} (0.099, 0.056 and 0.044 g.L^{-1}.h^{-1}).

**Conc.= Concentration*

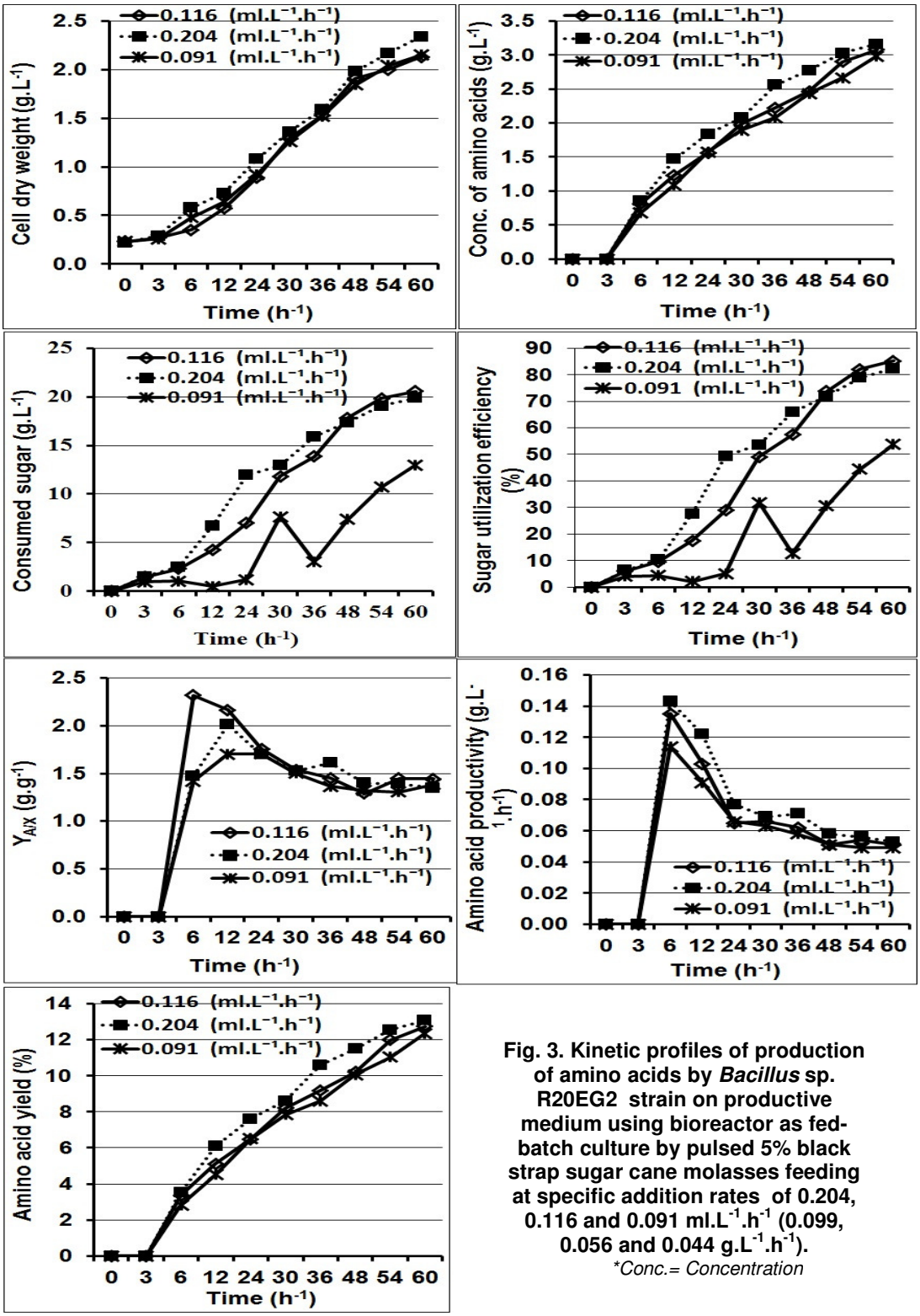

Fig. 3. Kinetic profiles of production of amino acids by *Bacillus* sp. R20EG2 strain on productive medium using bioreactor as fed-batch culture by pulsed 5% black strap sugar cane molasses feeding at specific addition rates of 0.204, 0.116 and 0.091 ml.L^{-1}.h^{-1} (0.099, 0.056 and 0.044 g.L^{-1}.h^{-1}).

**Conc.= Concentration*

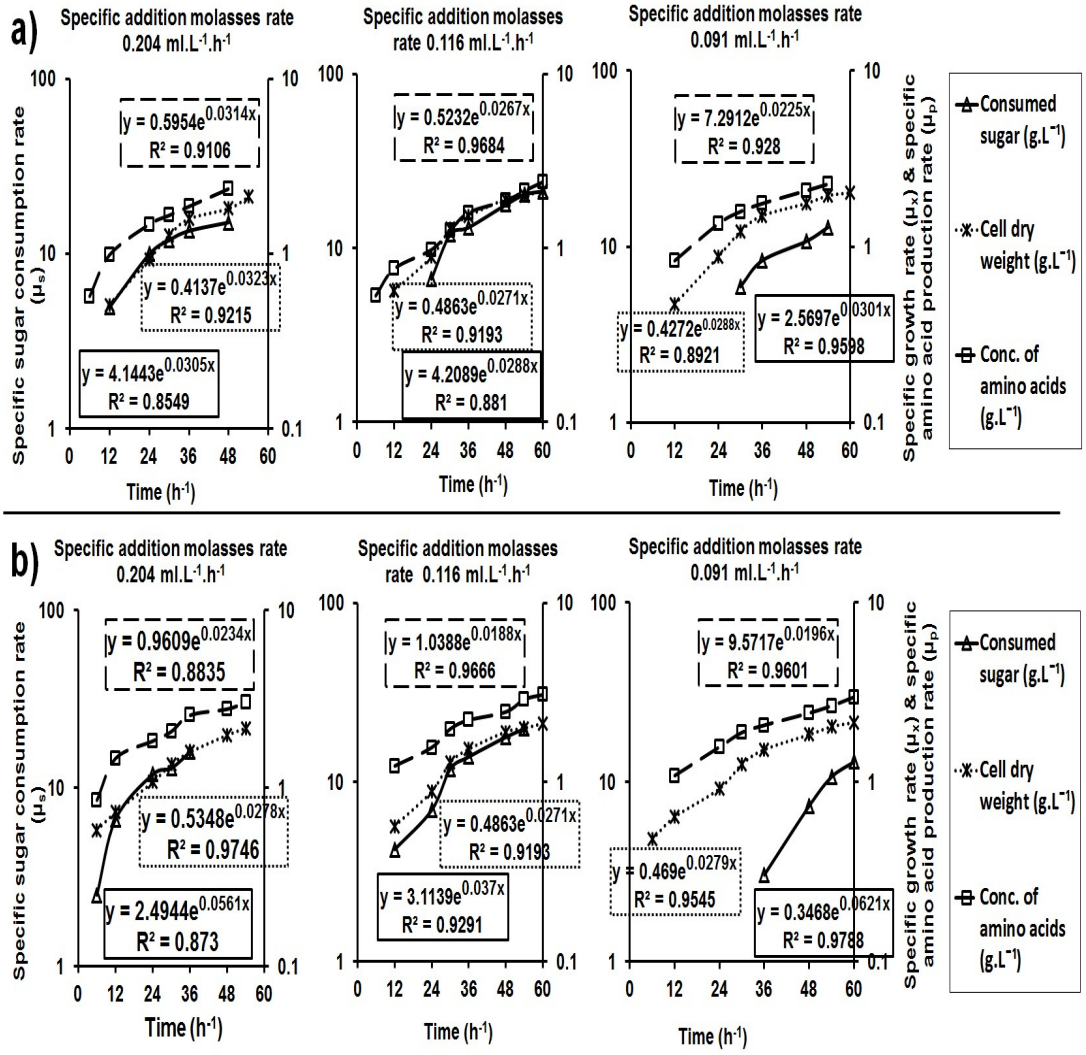

Fig. 4. The specific growth rate (μ_x), specific amino acid production rate (μ_p) and specific sugar consumption rate (μ_s) on productive medium using bioreactor as fed-batch culture by pulsed 5% sugar cane molasses feeding at specific addition rates of 0.204, 0.116 and 0.091 ml.L^{-1}.h^{-1} for:- a) *Bacillus* sp. R22EG1 strain b) *Bacillus* sp. R20EG2 strain

**Conc.= Concentration; The R^2 values for the trendlines between (cell dry weight, amino acid conc. & consumed sugar) and fermentation time*

amount of free amino acids (glutamic acid) was achieved by *Brevibacterium divaricatum* and *Bacillus methanolicus*. Glutamic acid production was also previously reported by Tarek and Mostafa [39], Zalán et al. [40] and Zareian et al. [41] for some of the LAB species such as *Lactobacillus paracasei*, *Lactobacillus* spp. and *Lactobacillus plantarum*. The level of glutamic acid produced by lactic acid bacteria was reported to be 68.7 mg/L [39], <25 mmol/L [40] and 1.032 mmol/L [Zareian et al. [41]. *R. leguminosarum* bv. *phaseoli* and bv. *trifolii* produced high amount of serine, glycine and alanine but *Mesorhizobium loti* strain U226 produced small amounts of serine, glycine, threonine, alanine, proline, tyrosine, valine, isoleucine and leucine [42].

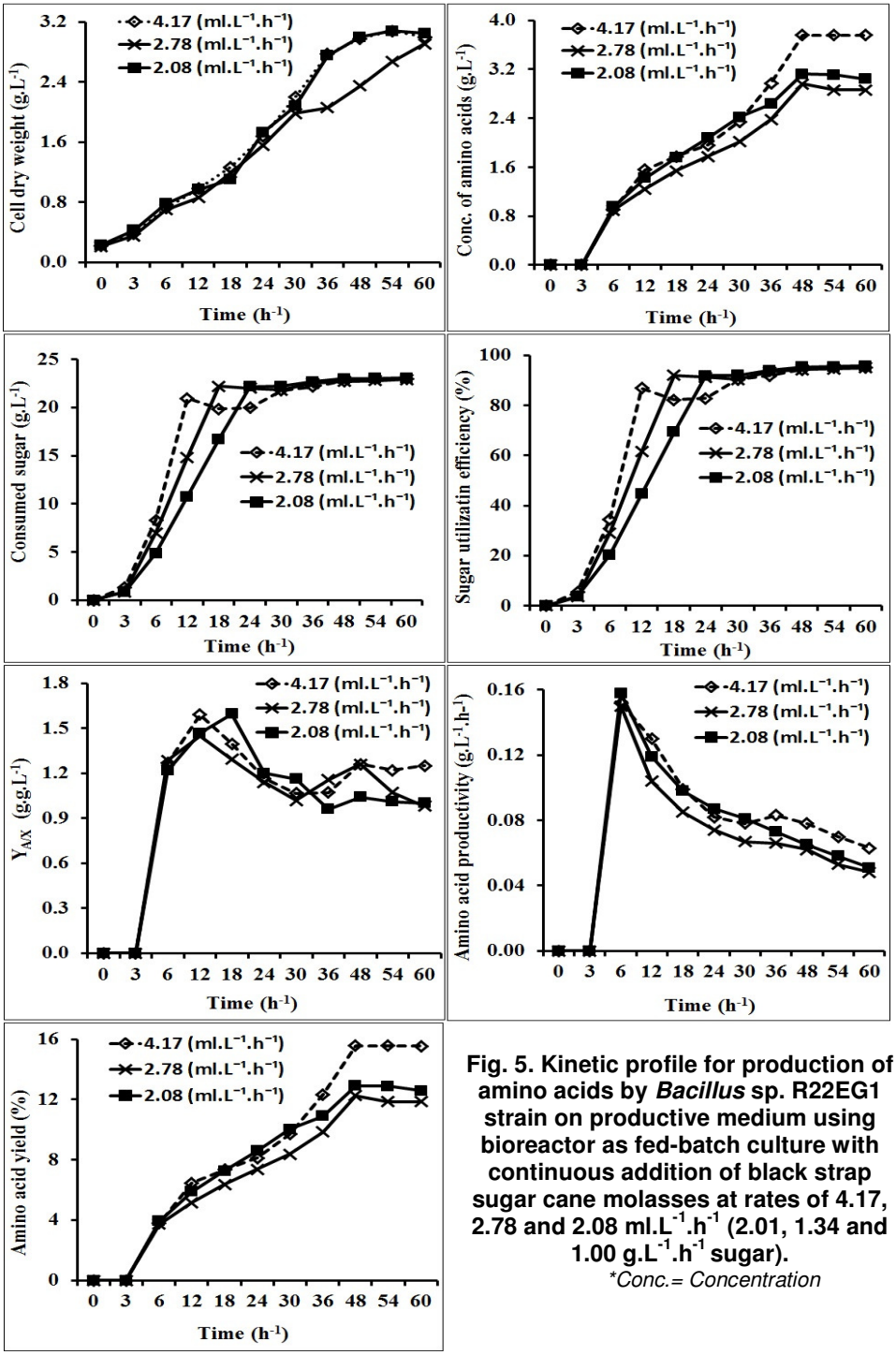

Fig. 5. Kinetic profile for production of amino acids by *Bacillus* sp. R22EG1 strain on productive medium using bioreactor as fed-batch culture with continuous addition of black strap sugar cane molasses at rates of 4.17, 2.78 and 2.08 ml.L^{-1}.h^{-1} (2.01, 1.34 and 1.00 g.L^{-1}.h^{-1} sugar).

**Conc.= Concentration*

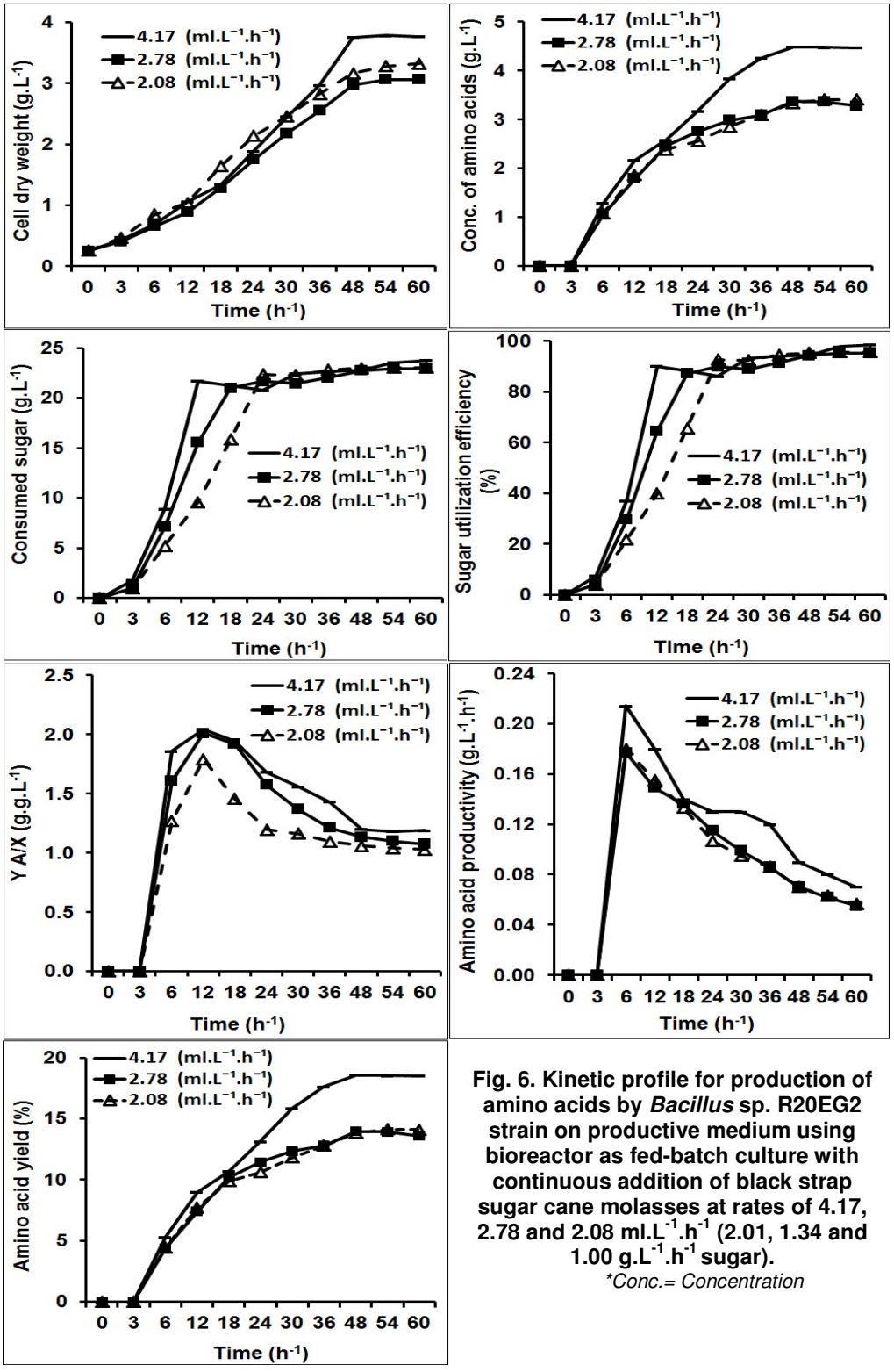

Fig. 6. Kinetic profile for production of amino acids by *Bacillus* sp. R20EG2 strain on productive medium using bioreactor as fed-batch culture with continuous addition of black strap sugar cane molasses at rates of 4.17, 2.78 and 2.08 ml.L^{-1}.h^{-1} (2.01, 1.34 and 1.00 g.L^{-1}.h^{-1} sugar).

**Conc.= Concentration*

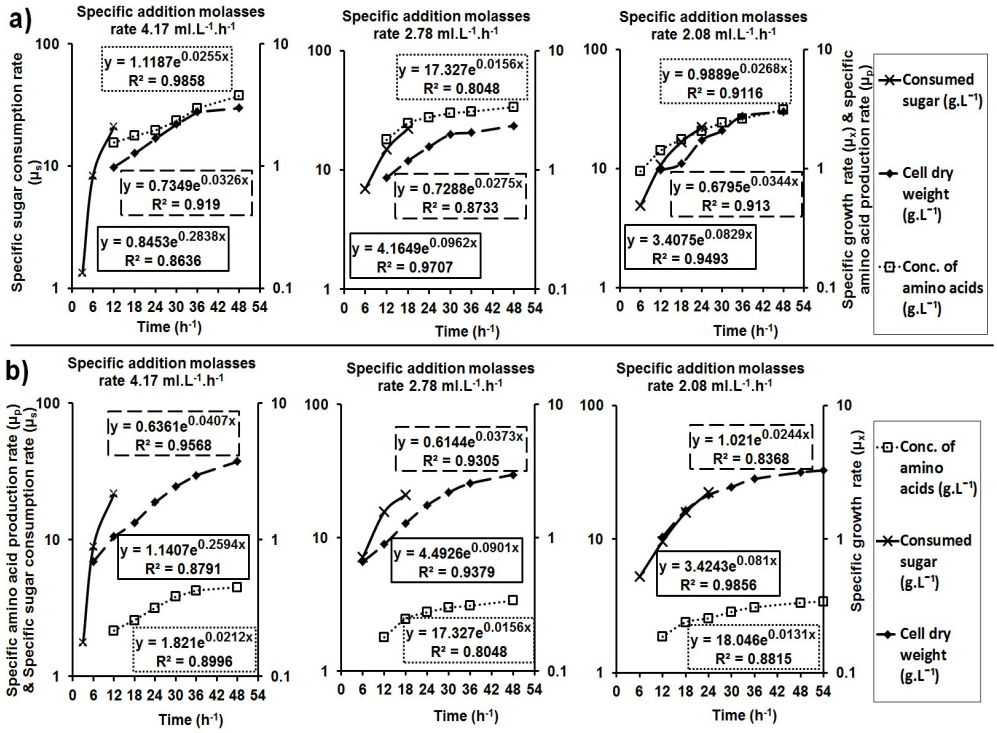

Fig. 7. The specific growth rate (μ_x), specific amino acid production rate (μ_p) and specific sugar consumption rate (μ_s) on productive medium using bioreactor as fed-batch culture with continuous addition of black strap sugar cane molasses at rates of 4.17, 2.78 and 2.08 ml.L^{-1}.h^{-1} for:- a) *Bacillus* sp. R22EG1 strain b) *Bacillus sp. R20EG2* strain

**Conc.= Concentration; The R^2 values for the trendlines between (cell dry weight, amino acid conc. & consumed sugar) and fermentation time*

Table 1. Identification of free amino acids produced by *Bacillus* sp. R22EG1 and R20EG2 strains using continuous fed-batch culture with addition rate 4.17 ml.L^{-1}.h^{-1} of black strap sugar cane molasses at 48 h

Amino acids	Amino acid contents mg/100ml	
	***Bacillus* sp. R22EG1 strain**	***Bacillus* sp. R20EG2 strain**
Aspartic acid	36.70	48.72
Threonine	11.29	13.65
Serine	13.41	17.38
Glutamic acid	67.40	85.62
Glycine	14.96	17.45
Alanine	13.31	17.26
Valine	10.29	14.81
Isoleucine	10.32	14.64
Leucine	17.58	23.76
Tyrosine	12.66	18.67
Phenylalanine	17.39	25.10
Histidine	11.20	15.08
Lysine	20.60	32.00
Arginine	16.67	22.90
Proline	17.43	29.06
Cysteine	5.70	7.60
Methionine	3.54	4.74

4. CONCLUSION

Here in this study, the maximum production of amino acids from continuous fed–batch by *Bacillus* sp. R22EG1 and R20EG2 strains was recorded as 3.76 and 4.49 $g.L^{-1}$ with continuous feeding at 4.17 $ml.L^{-1}.h^{-1}$ (2.01 $g.L^{-1}.h^{-1}$ sugar) at 48 h, respectively. These results were higher, approximately 1.53 & 1.42 fold compared to pulsed feeding and 1.64 & 1.59 fold higher than batch fermentation by *Bacillus* sp. R22EG1 and R20EG2 strains, respectively. The maximum content of free amino acids was glutamic acid, approximately 67.40 and 85.62 mg/100 ml, for *Bacillus* sp. R22EG1 and R20EG2 strains, respectively.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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