The Role of Amino Acids in the Amplification and Quality of DNA Vectors for Industrial Applications.

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Abstract

In this study, we have demonstrated that the type and feeding regimen of amino acids have a significant impact on the quality as well as the quantity of DNA vectors produced. Nutrient pool and factorial design experiments were carried out in order to identify the amino acids involved in increased biomass and induction of plasmid amplification. Leucine, glycine, and histidine were responsible for increased biomass and leucine starvation in the presence of histidine was implicated in plasmid amplification. Supercoiling of the plasmid was optimised using a dual feeding strategy. As a result of this, a fed-batch fermentation strategy for the production of a 6.9kb plasmid, pSVß, in *Escherichia coli* DH5α was developed. In batch fermentation, a maximum plasmid yield of 39.4mg/L equivalent to 11.3mg/g DCW was achieved with casein hydrolysate limitation. 90% of plasmid was in the supercoiled form after 31h of fermentation but only remained so for a short period, leading to a very brief window for harvesting cells at scale.

Subsequently, a fed-batch fermentation using a dual feeding strategy was employed. A mean maximum plasmid yield of 44 mg/L equivalent to 9.1 mg plasmid/g DCW was achieved. After 25h, 90% of plasmid was in the supercoiled form and remained at this level for the remaining 10h of the fermentation, allowing adequate time for the harvesting of cells without the loss of supercoiling of product. This study emphasized that optimizing fermentation strategy and identifying the essential nutrients are beneficial for bioprocessing of plasmid DNA for therapeutic applications.

Introduction

Gene therapy and DNA vaccination may both be achieved by the administration of non-viral vectors, of which the production and quality is the concern of this study. The fact that there are currently over 2463 clinical trials worldwide in gene therapy of which

about 17% are plasmid-based constituting the third largest number after the use of retroviruses and adenoviruses[1] indicates a renewed interest in addressing factors which affect the manufacture of plasmids.

Literature holds several reports on fermentation strategies for the production of non-viral DNA vectors[2-6]; however, the focus of the majority of these reports has been on the quantity and not the quality of the product with no distinction made between the different types, e.g. supercoiled versus open circular. The current perception is that supercoiled (SC) pDNA is the best form for administration[7]. Recently a report of high cell density batch culture was made in which approximately 50 mg/L of plasmid was obtained with 90 % being in the supercoiled form[8]; in another, volumetric yields up to 1500mg/L of plasmid was obtained through a fed-batch system with optimized plasmid backbones, resulting in 93% plasmid supercoiling after a temperature induction step[9]. Also, plasmid yield has been improved by supplementing media with nitrogen sources such as ammonium chloride and amino acids as the nitrogen is required in the biosynthesis of purine and pyrimidine nucleotides[8,56,57]. It is worth noting that we have previously demonstrated that the quality and quantity of DNA is host strain/plasmid dependent[10]. We have also previously discussed the effects of different fermentation modes on the quality of alkaline lysate extracts and plasmid supercoiling[11].

A further study[12] described a process for producing structurally stable supercoiled plasmid dimers and trimers. Such plasmid concatamers are of interest because they may have increased transfection efficiencies when used as gene therapy vectors. FDA guidelines state that a specification for the minimum level of supercoiling, in the final product, should be made[13]. A fermentation should, therefore, aim to yield pDNA in the mainly supercoiled form and contaminants such as genomic DNA (gDNA) should be

minimised. Our group[14] was one of the first to demonstrate that the fermentation medium could have a profound effect on plasmid quality and contaminant levels. A semi-defined medium, consisting of salts, glucose, and casein hydrolysate, was shown to support a higher cell density and plasmid stability than the complex medium, LB, and plasmid extracted from cells grown on semi-defined medium was of a higher quality with respect to supercoiling and gDNA level. There are other reports of fermentation strategies for the production of pDNA; A summary of those reported is given in reference[50]. Wang *et al*.[15] showed that plasmid stability was higher in a defined medium in comparison to two complex media. These authors were also able to obtain a higher specific plasmid yield from cells grown on their defined medium than those grown on LB medium. Therefore the choice of medium for a pDNA fermentation must be carefully made to maximise the yield of SC pDNA and facilitate downstream processing. In general defined synthetic media are preferred to complex or semi-defined media because they reduce inherent process variability leading to a reproducible process in which nutrient composition is well defined and easily monitored. Also, the use of synthetic media avoids the use of materials which pose a transmissible spongiform encephalopathy risk[16].

Specific plasmid yield (mg plasmid/g DCW) may be improved by inducing plasmid amplification, for example by the addition of chloramphenicol to the fermentation medium[17] or by temperature shift[2,9]. There have been some attempts at increasing specific plasmid yield by high cell density culture usually through plasmid engineering[51,52]. For therapeutic pDNA production, chloramphenicol addition is not a suitable method because of the need to demonstrate complete removal from the final product. Temperature shift induction may be costly at large scale and therefore not suitable. Hecker *et al.,*[18] suggested that plasmid amplification may be induced by amino acid starvation, which is potentially preferable at industrial scale as it does not

involve the introduction of additional compounds to the fermentation medium. Amplification has been demonstrated for a wide range of plasmids[19-23]. Hofmann *et al*[24] were able to improve specific plasmid yields in batch and fed-batch fermentations by employing amino acid starvation, but the quality of the plasmid obtained and the impact of amino acid starvation on this has not been reported.

In this paper, we have investigated a strategy in which it is feasible to increase the quantity of DNA vectors while producing a high quality of the product (level of supercoiling) for an extensive period.

Materials and methods

Plasmids and bacterial strain

pSVβ (Promega Corp. Woods Hollow Road, MA, USA) a plasmid of 6.9 kb with an ampicillin resistant marker and a high copy number pUC-based origin. It was transformed into *Escherichia coli* DH5α (F^- φ80d*lac*ZΔM15, Δ(*lac*ZYA-*arg*F), U169, *end*A1 *rec*A1 *hsd*R17(r_k- m_k+) *deo*R *thi*-1 *sup*E44 λ^-*gyr*A96 *rel*A1) using standard techniques, details of which are given elsewhere[25]. The host and recombinant strains were maintained in 20 % ($^V/_V$) glycerol at –85 °C. Previously, this plasmid/host strain combination has been shown to be one of the better-performing systems for SC-DNA production[10].

Growth media

Plate medium was LBM agar which consisted of, per litre, mycological peptone (10 g; Oxoid, Basingstoke, Hampshire, UK), yeast extract (5 g; Oxoid), sodium chloride (10 g) agar bacteriological (10 g; Oxoid). LBM-X-gal agar was used to enhance X-gal colour development *(26).* X-gal (40 mg/L; BioGene, Kimbolton, Cambridgeshire, UK) was dissolved in dimethylformylamide and added to cooled autoclaved LBM agar. Batch cultures were grown on defined medium (DM) which consisted of, per litre, D-glucose (10 g), magnesium sulphate heptahydrate (1.2 g), ammonium sulphate (4 g), potassium dihydrogen orthophosphate (13.3 g), citric acid (1.7 g), EDTA sodium salt (8.4 mg), cobalt chloride hexahydrate (2.5 mg), manganese sulphate (15 mg), copper sulphate dihydrate (1.5 mg), boric acid (3 mg), sodium molybdate dihydrate (2.5 mg), zinc chloride (13 mg), iron (III) citrate (100 mg) and thiamine hydrochloride (4.5 mg; Sigma, Poole, Dorset, UK). (4.5 mg; Sigma, Fancy Road, Poole, UK). The pH of DM was adjusted to 6.3 before sterilisation by addition of 4 M sodium hydroxide. All chemicals were obtained from BDH (Poole, Dorset, UK) unless otherwise stated.

Where selective conditions for plasmid-containing cells were required, cultures were supplemented with ampicillin (100 mg/L; Sigma). For 7 L fermentations, casein hydrolysate (Oxoid) was added to Defined medium to a final concentration of 10 g/L to form SDCAS medium.

Culture conditions

All cultures were grown at 37 °C. Small-scale cultures (10 mL) were grown in McCartney bottles and agitation was provided by shaking horizontally at 200 rpm in a rotary shaking incubator (G25 incubator shaker, New Brunswick, USA). 20 µL of overnight seeded culture was inoculated to each bottle and cultures were grown for 21.5 h. For nutrient pool experiments DM was supplemented according to Holiday (27). Design Expert 5 (DX-5, Statease Corp., Minneapolis, USA) was used to create the quarter fraction duplicated factorial design, and DM medium was supplemented accordingly (see supplementary material, Table 1). Fermentations were carried out in a 7 L (5.5 L working volume) LH210 series bioreactor (Adaptive Biosystems Ltd., Progress Business Park, Luton, UK) which was aerated at 5.5 L/min. Oxygen levels were monitored using an Ingold polarographic probe (Mettler-Toledo Ltd., Beaumont Leys, Leicester, UK) and maintained at 30 % of saturation by an automatic stirrer. pH was maintained at 6.3 ± 0.02 by automatic additions of 4 M NaOH and 4 M phosphoric acid. Foaming was controlled automatically by the addition of 100 % Polypropylene Glycol 2025. Exit gas analysis of CO_2 and O_2 was carried out by mass spectrometry (VG Gas Analysis Systems Ltd., Middlewich, Cheshire, UK). Online data was logged by Propack data logging and acquisition software (Acquisition Systems, Fleet, Hampshire, UK). 40% ($^{W}/_{V}$) D-glucose was fed exponentially to achieve a growth rate of 0.1 h^{-1} using two Watson Marlow 101U pumps (Watson Marlow, Falmouth, Cornwall, UK). The feed profile was calculated by Propack according to Gregory and Turner[28] based on manually input biomass values. For amino acid fed cultures, the amino acid

feed which consisted of, per litre; leucine (0.64 g; BDH), glycine (0.56 g; Sigma) and histidine (0.17 g; BDH) was fed linearly at 60 mL/h.

Sampling and analysis

Sampling was carried out at 1 h intervals and various measurements were made. Optical density was measured at 625 nm in a Pharmacia Biotech Ultrospec 2000 (Pharmacia Biotech, Buckinghamshire, UK). Wet cell pellets and culture supernatants were frozen at –20 °C for retrospective analysis.

Wet and dry cell pellets

To obtain wet cell pellets triplicate 5 mL aliquots of culture were centrifuged at 3,500 rpm (GS6R; Beckman, High Wycombe, Buckinghamshire, UK) for 20 min at 4 °C in sterile universals. The supernatant was transferred to clean tubes and cell pellets were washed in 5 mL of sterile reverse osmosis (RO) water and recentrifuged. Pellets were resuspended in approximately 1 mL of residual supernatant, transferred to sterile 2.2 mL microtubes and pelleted at 13,000 rpm for 3 min (Biofuge 13, Heraeus, Essex, UK). For dry cell weight (DCW) determination triplicate 5 mL aliquots of culture were centrifuged at 3,500 rpm for 20 min at 4 °C in pre-weighed McCartney bottles. Pellets were washed in 5 mL of sterile RO water and recentrifuged, before being dried at 80 °C for 24 h.

D-glucose assay

D-glucose concentration in culture supernatants was determined using the dinitrosalicylic acid (DNS) reducing sugar assay[29]. Where necessary supernatants were diluted in RO water to bring them into the range of the calibration curve. 500 µL of culture supernatant was mixed with 500 µL of DNS reagent in glass test tubes which were covered with foil to prevent evaporative loss. Samples were placed in a boiling

water bath for 5 min to allow colour development. Samples were chilled on ice and 4 mL of RO water was added. Absorbance at 540 nm was measured and compared to a calibration curve constructed from D-glucose in the range of 0 - 4 g/L to determine residual D-glucose concentration.

Amino acid assay

Total amino acid levels in culture supernatants were measured by absorbance at 570 nm after reaction with ninhydrin. 500 µL of culture supernatant was mixed with 500 µL of 60 % perchloric acid in microfuge tubes to precipitate proteins. Tubes were centrifuged for 3 min at 13,000 rpm and supernatants were transferred to clean tubes. Supernatants were diluted $^{1}/_{100}$ in RO water and 1 mL was mixed with 1 mL ninhydrin reagent (Sigma) in glass test tubes. Tubes were covered with foil to prevent evaporative loss and transferred to a boiling water bath for 15 min. Samples were placed on ice and 3 mL RO water was added. Absorbance at 570 nm was measured against a blank prepared from RO water and treated in the same manner as the samples. Different amino acids form different coloured products when reacted with ninhydrin. The amino acid levels were therefore expressed in terms of absorbance levels and not absolute amino acid concentrations.

Percentage of plasmid-containing cells

Culture samples were appropriately diluted in sterile 0.9 % ($^{W}/_{V}$) sodium chloride and plated onto LBM-X-gal agar. Plates were incubated at 37 °C for 24 h. The percentage of plasmid-containing cells was calculated from the number of blue colonies divided by the total number of colonies grown on LBM-X-gal plates multiplied by 100.

Plasmid DNA extraction

pDNA was extracted as described by Birnboim and Doly[30]. Cell pellets were defrosted on ice and resuspended in 300 µL TE buffer (10 mM Tris HCl pH 8, 1 mM EDTA pH 8), containing RNase A at 50 µg/mL, by vortexing. 300 µL alkaline lysis buffer (1% SDS, 200 mM NaOH) was added to the cell suspension and used to lyse the cells and lysis was allowed to proceed for 4 min. 300 µL ice-cold 3 M potassium acetate (pH 5.5) was added to each tube and tubes were incubated on ice for 30 min. The neutralised lysate was clarified by centrifugation at 13,000 rpm for 5 min. The supernatant was transferred to clean tubes and 700 µL of isopropanol was added to precipitate DNA. Precipitated DNA was pelleted at 13,000 rpm for 5 min and the supernatant was discarded. After air-drying overnight DNA pellets were resuspended in 150 µL of TE buffer.

PicoGreen plasmid analysis

Total pDNA yield was determined by PicoGreen assay, based on the method of Noites *et al.,*[31]. PicoGreen reagent and pDNA samples were diluted $^{1}/_{400}$ in TE buffer to give working solutions. Equal volumes (100 µL) of diluted sample and PicoGreen working solution were mixed in a 96-well plate, incubated and protected from light at room temperature for 3 min. Fluorescence was read in a 96 well microtitre plate fluorimeter (Fluorocount, Packard Bioscience, Berkshire, UK) controlled through plate reader software (Packard Bioscience) at excitation and emission wavelengths of 485 nm and 530 nm, respectively. pDNA concentration was determined by comparison of fluorescence with a standard curve constructed using Qiagen purified (Qiagen tip 20, Qiagen, West Sussex, UK) pDNA in the range of 30 – 1000 ng/mL.

Gel electrophoresis

Gel electrophoresis was used to determine the percentage of SC plasmid obtained from fermentation. 0.7 % ($^{w}/_{v}$) agarose gels were made up in 1 x TBE buffer (90 mM Tris, 90 mM boric acid, 1 mM EDTA). Gels were run at 80 V, 40 mA in 1 x TBE for 2 h. DNA in gels was visualised by staining in ethidium bromide (0.5 µg/mL) for 20 min. Gels were scanned using UVP 5000 Gel Documentation System (Ultra Violet Products Ltd., Cambridge, UK). Densitometry plots were obtained using UVP Gelbase (Ultra Violet Products Ltd.). A correction factor of 1.36 was applied to peak areas to correct for the increased binding efficiency of ethidium bromide to open circular pDNA and gDNA[32]. The percentage of SC pDNA was calculated as the proportion of SC pDNA to total pDNA multiplied by 100. The total pDNA was calculated as the sum of all peak areas identified as plasmid.

Results and discussion

Effect of casein hydrolysate concentration on final biomass and specific plasmid yields

Triplicate 10 mL cultures were grown on DM supplemented with casein hydrolysate to final concentrations of 0 %, 0.1 % or 1 % ($^{W}/_{V}$) for 21.5 hours. The biomass and plasmid yield values are shown in Figure 1. A decrease in casein hydrolysate concentration was associated with decreased biomass yield but increased specific plasmid yield. This observation was consistent with plasmid amplification by amino acid starvation. Silva *et al*[55] reported a similar occurrence when tryptone was used as the only amino acid source. A low tryptone concentration yielded the highest specific plasmid yield. In cultures with no casein hydrolysate supplement, cells became starved of amino acids rapidly following inoculation and hence demonstrated low biomass and high specific plasmid yields. In cultures supplemented with 0.1 % ($^{W}/_{V}$) casein hydrolysate cells became starved of amino acids at a later stage and hence demonstrated higher biomass, but lower specific plasmid yields due to the reduced amplification time.

The cultures supplemented with 1 % ($^{W}/_{V}$) casein hydrolysate demonstrated high biomass and low specific plasmid yields, it is likely that these cultures did not become starved of amino acids during the duration of culture and hence did not show any plasmid amplification. These initial results suggested that amino acid starvation was a viable method for plasmid amplification. The high specific plasmid yield observed is because plasmid amplification is promoted in *relA* mutant strains of *E. coli* which DH5α is[53]. During amino acid starvation, *relA* mutant strains of *E. coli* are relaxed rather than the stringent response observed in the wild-type strains. The stringent response of wild-type strains includes inhibition of plasmid replication promoter and hence suppression of plasmid replication[54].

5 L batch fermentation

A typical OUR profile for the batch fermentation is shown in Figure 2 and biomass, plasmid and nutrient profiles are shown in Figure 3. OUR began to drop rapidly at 17.5 hours. This drop indicated D-glucose exhaustion which was confirmed by D-glucose assay (see Figure 3). Biomass became steady at a mean value of 3.5 DCW g/L by 23 hours. The biomass level was comparable to that previously reported[11].

Plasmid yield increased in line with growth and reached 20 mg/L by 16 hours. By 23 hours it had reached 40 mg/L but fell to a mean value of 25 mg/L by 25 hours. Specific plasmid yield increased from 6.3 mg/g DCW at 20 h to 11.3 mg/g DCW by 23 hours. After 23 hours specific plasmid yield dropped back to 7.1 mg/g DCW. The increase in volumetric and specific plasmid yields was attributed to plasmid amplification due to amino acid limitation. However, the fall in plasmid yields following amplification demonstrated that amplified plasmid levels could not be maintained. A decline in plasmid yield following amplification was reported by Hofmann *et al*[24], although the rate of decline was much lower than observed here. The work reported by Hofmann et al[24] was conducted at 32 °C whereas this work was conducted at 37 °C. Elevated temperatures have been shown to increase the rate of DNA degradation following plasmid amplification in amino acid-starved *E. coli*[33]. The difference in temperature between this work and that reported by Hofmann *et al*[24] may explain the increased rate of degradation.

The level of plasmid in SC form declined steadily during the fermentation and reached a mean minimum value of 40 % of total plasmid yield after 25 hours. This level was maintained until 28 hours when it increased sharply and reached a maximum value of 90 % by 31 hours for 1 hour. A decline to a value of 70 % by 34 hours, was then observed. The level of supercoiling observed to 25 hours agrees with those previously

reported[11]. In previous work the fermentation was harvested after 24 hours, so the increases in supercoiling after 24 hours were not observed.

Although plasmid yield was increased via amplification, only 50 % of the resultant plasmid was in the SC form. Given the downstream challenges reported in the separation and purification of the SC form of plasmid DNA[33-41], it was evident that further optimisation was required, to increase the level of supercoiled plasmid, to be considered for use at harvest time[41]. Following further culture incubation, the percentage of plasmid in the SC form increased and reached 90 % by 31 hours but was only maintained for 1 hour. Therefore, the best point of harvest for this fermentation was between 30 and 31 hours when the percentage of plasmids in the SC form was 90 %. In a production environment, a harvest window of 1 hour is very tight and may not be easily implemented. Therefore, a fermentation in which a high percentage of supercoiling is maintained for an extended period would be preferable.

Nutrient pool experiments

The results of the batch fermentation demonstrated that pDNA obtained from amplification induced by amino acid starvation was not of a suitable quality, with respect to supercoiling, for further processing as a gene therapy product. An alternative, fed-batch strategy was chosen as a method of improving plasmid yield. The intent was to feed D-glucose and a defined amino acid mixture to increase biomass and prevent induction of plasmid amplification. In this manner volumetric plasmid yield would be improved owing to the increased biomass and plasmid would be of suitable quality for gene therapy due to the prevention of amplification.

The first step in developing such a fed-batch strategy was to identify the amino acids, which were involved in plasmid amplification, and other nutrients that could aid

biomass increase. Nutrient pool experiments were carried out to identify compounds to carry forward into a factorial design.

Triplicate 10 mL cultures of host and recombinant strains were grown on DM supplemented according to Holiday[27]. Control cultures were grown on DM with no supplements. Final biomass and plasmid yield values were determined. The results of this investigation are shown in Table 2. The details of the nutrient pool experiment can be found in the supplementary material. The identified nutrients were carried over to determine which nutrient had the greatest influence on the biomass and plasmid yield using factorial design.

Factorial design

Having identified nutrients which contributed to an increase in biomass, a screening technique was required to determine which nutrients had the greatest influence on biomass and if any of the identified nutrients were involved in plasmid amplification. A fractional factorial design was therefore chosen to screen the nutrients identified in the nutrient pool investigation. In addition to the six nutrients identified in the nutrient pool study, threonine was used as a negative control. Threonine was not identified in the nutrient pool study but had previously been implicated in plasmid amplification[23]. A two-level, duplicated quarter fraction factorial design, requiring 64 runs, was chosen and created using Design Expert 5 (DX-5) software. The design allowed high order effects and interactions to be identified using an experimentally manageable number of cultures.

The analysis showed that high concentrations of glycine, leucine and histidine were expected to lead to increases in biomass, while a high concentration of threonine was expected to inhibit biomass increase. This observation indicated that glycine, leucine and histidine should be considered for use in a fed-batch strategy to increase biomass yield.

Individual negative effects were not identified for specific plasmid yield, but a two-factor interaction between leucine and histidine was identified. When two compounds interact the outcome of the interaction is dependent on the concentrations of the two compounds. The data indicated that the highest specific plasmid yield was expected when leucine was starved in the presence of a high histidine concentration. The requirement of histidine in plasmid amplification is not surprising as the imidazole ring of histidine, is implicated in the synthesis of purine nucleotides which is necessary for DNA synthesis[56,57]. This observation indicated that leucine should be used in a fed-batch strategy to prevent plasmid amplification.

Fed-batch fermentation

Initially, an amino acids feed composed of leucine (6.38 g/L), glycine (5.6 g/L) and histidine (1.68 g/L), based on amino acid utilisation rates reported by Gschaedler and Boudrant[42], was fed at 60 mL/h. This feed proved to be too concentrated. Cells died rapidly following commencement of the feed, demonstrated by a rapid fall in the number of cells (measured as CFUs) (data not shown). A $^{1}/_{10}$ dilution of this feed was subsequently used, with the feed rate being maintained. A typical OUR profile for the fed-batch fermentation is shown in Figure 4 and typical biomass, plasmid and nutrient profiles are shown in Figure 5.

At 15 h OUR dropped rapidly indicating D-glucose exhaustion, which was confirmed by the DNS reducing sugar assay (see Figure 5) and the exponential D-glucose feed was started. OUR increased rapidly to a value of 19.6 mmol/L/h and continued to climb, reaching a new maximum level of 25.6 mmol/L/h at 20.7 h when the linear amino acids feed was started. The start of feeding was based on observations of nutrient limitation during the batch fermentation. A maximum value of 26.6 mmol/L/h was achieved at 22 hours. A decline to 7.4 mmol/L/h at 24.2 h was then observed. From 24.2 to 28.2 hours oxygen uptake rate increased to 15 mmol/L/h/L/h then dropped to 11 mmol/L/h and remained steady until harvest at 35 hours.

Biomass climbed steadily and reached a value of 3.7 g/L DCW by 15 hours, which was slightly higher than previous batch fermentations. Following commencement of the exponential D-glucose feed, biomass continued to climb steadily and reached a maximum value of 4.9 g/L DCW at 21 hours which was maintained for the remaining 14 hours of the fermentation.

Plasmid yield reached a mean maximum value of 44 mg/L at 20.7 hours which was maintained for the remainder of the fermentation. Specific plasmid yield remained constant at a mean value of 9.1 mg/g DCW, no amplification was observed.

The percentage of plasmid in the SC form remained at a mean value of 50 % until 22 hours when it started to increase. By 25 hours, 90 % of plasmid was in the SC form and remained at this level until harvest at 35 h. This contrasts with the batch fermentation where high levels of supercoiling were maintained for only 1 hour.

The decline in OUR observed between 21.7 and 24.2 hours coupled with the steady biomass suggests that the culture had become starved of a further nutrient necessary for growth. That no plasmid amplification was observed during this time indicates that

the amino acid feed was sufficient to prevent amino acid starvation but not sufficient, in combination with the D-glucose feed, to increase biomass. Besides D-glucose, the major components of SDCAS are ammonium sulphate, potassium dihydrogen phosphate and casein hydrolysate. The starved nutrient is likely to be one of these components. An alternative theory is that acetic acid may have reached an inhibiting concentration. Measurements of nutrient and acetic acid concentrations in the culture were not made so neither of these hypotheses may be proven or disproven. Further work is necessary to determine which the case is.

If growth ceased due to starvation of a further nutrient(s), then identification of this nutrient(s) would allow continued growth with an associated increase in volumetric plasmid yield. As it currently stands our medium is semi-defined and uses a bovine-derived component, casein hydrolysate, as an amino acids source. The ideal case for future investigation would be to replace the casein hydrolysate with a defined amino acid mixture. Wang *et al.*[15] developed a fully defined medium using a glucose and salts base supplemented with 6 amino acids. In batch culture, this medium was able to support cell densities and plasmid yields similar to those reported in this study. The addition of ribonucleotides to their medium led to a doubling of specific plasmid yield. These results suggest that we could move to a fully defined medium without detriment to plasmid yields. Further investigation is required to determine a suitable defined medium and its impact on plasmid yield and quality. The increase and steadying in OUR observed from 24.2 hours is likely to be due to cells entering a steady state of maintenance. Once biomass became steady, the D-glucose feed effectively became linear, due to the manual correction of Propack's estimated biomass values. All available D-glucose was taken up by the cells, indicated by the very low levels of residual D-glucose (see Figure 5), suggesting that it was used in maintenance activity. Schmidt *et al*.,[43], using a feeding strategy and maintaining the growth rate at 0.15/h

were able to achieve a biomass yield of 50 g/L DCW and a plasmid yield of 225 mg/L. This suggests that if we were able to identify the starved nutrient(s) during the feeding stage of the fermentation, we would be able to achieve much higher biomass yields and hence plasmid yield. This requires further investigation and impact on plasmid supercoiling would require assessment.

The fed-batch fermentation achieved a 40 % biomass increase over the batch fermentation. The specific plasmid yield in the fed-batch fermentation was maintained at an average of 9.1 mg/g DCW and no amplification was observed. In the batch fermentation, a specific plasmid yield of 7.1 mg/g DCW was achieved prior to amplification. Hence the fed-batch fermentation achieved a 28 % higher specific plasmid yield than the batch fermentation. It is unclear why the specific plasmid yield differed between the two fermentation strategies. However, the casein hydrolysate used in the fed-batch fermentation was from a different batch to that used in the batch fermentation. The two lots were obtained from different sources[44] and hence contained different nutrient levels which may have caused the differences in plasmid yield.

The amino acids feed did not increase the biomass yield over that obtained from feeding D-glucose, however, within 5 h of initiating the amino acids feed, the percentage of plasmid in the SC form had increased to 90 % and was maintained for the remaining 10 hours of the fermentation. This was a great improvement over the batch fermentation where high levels of supercoiling were only maintained for 2 hours. Changes in intracellular ATP/ADP ratios, associated with changing environmental conditions, have been reported to lead to increases in plasmid supercoiling[45,46]. It is possible that the change in environment, associated with the loss of a nutrient from the medium and a shift in the growth phase, led to changes in the intracellular ATP/ADP

ratio which led to the increase in supercoiling observed in both the batch and fed-batch fermentations. It seems that feeding amino acids and D-glucose for cellular maintenance allowed the level of supercoiling to be sustained. The maintenance of both plasmid yield and plasmid supercoiling at constant levels for an extended period of time creates a wider window of opportunity for harvest, meaning that harvest time is not critical to product quality.

Conclusion

We have demonstrated at laboratory and fermentation scale that amino acid starvation is a viable method for increasing specific plasmid yield. However, maximum plasmid yield and maximum plasmid supercoiling did not occur simultaneously, presenting a potential challenge for large scale plasmid production. A fed-batch strategy employing an exponential D-glucose feed and a linear amino acids feed were developed. This fed-batch strategy prevented plasmid amplification and resulted in a biomass increase over the batch fermentation. Plasmid was produced at mean levels of 44 mg/L and 9.1 mg/g DCW and was maintained at 90 % SC for 10 hours allowing a comfortable window for the harvesting of cells containing high quality (high supercoiled plasmid level) product. This is an improvement over our previous report in which only an exponential glucose feed was employed, and the final plasmid supercoiling was 50%.

In recent studies utilising various fermentation strategies, specific plasmid yields of up to 51mg/g DCW have been reported[9,47,48,49]. Although these reported specific plasmid yields are significantly higher than what was obtained in this study, further optimisation of our fed-batch plasmid amplification strategy may exert additional gains in terms of plasmid productivity, while maintaining high levels of plasmid supercoiling for an extensive period of time. Further work may involve determining the rate of amino acid

utilisation during feeding of amino acids in order to understand the shift in the ratio of plasmid species. Such an understanding may allow fine-tuning of the process. Alternatively, it may be worthwhile to determine the optimum host strain-plasmid combination for this system as the choice of host strain and plasmid has been demonstrated to have a significant effect on plasmid quality and quantity[10].

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List of Abbreviations

CFU	Colony forming unit
DCW	Dry cell weight
DM	Defined medium
DNA	Deoxyribonucleic acid
DNS	Dinitrosalycylic acid
FDA	Food and Drug Administration (USA)
gDNA	Genomic DNA
LBM	Modified Luria Bertrani medium
OD625	Optical density at 626 nm
OUR	Oxygen uptake rate
pDNA	Plasmid DNA
RNase A	Ribonuclease A
RO	Reverse osmosis
SC	Supercoiled
SDCAS	Semi defined medium with casein hydrolysate
SDS	Sodium dodecyl sulphate
X-gal	5-bromo-4-chloro-3-indolyl -D-galactopyranoside

Table 2. A; Biomass production of *E. coli* DH5a B; Biomass and plasmid production of E. coli DH5a pSVb grown on nutrient pools.

A

Pool	1	2	3	4	5	6	OD_{625} % of control
7	***Adenine***	Biotin	Phenylalanine	Alanine	Arginine	***Leucine***	230
8	***Hypoxanthine***	Folic acid	Serine	Cysteine	Ornithine	***Glycine***	186
9	Cytosine	Pantothenic acid	Tryptophan	Threonine	Aspartic acid	Isoleucine	96
10	***Guanine***	Pyridoxine	Tyrosine	Sodium thiosulphate	Proline	***Histidine***	219
11	/	Thiamine	P-amino benzoic acid	Methionine	Glutamic acid	Lysine	108
12	Uracil	Riboflavin	Nicotinic acid	Choline	Inositol	Valine	9
OD_{625} % of control	280	114	116	118	116	163	

B

Pool	1	2	3	4	5	6	OD_{625} % of control	Plasmid % of control
7	***Adenine***	Biotin	Phenylalanine	Alanine	Arginine	Leucine	158	35
8	***Hypoxanthine***	Folic acid	Serine	Cytosine	Ornithine	Glycine	130	91
9	Cytosine	Pantothenic acid	Tryptophan	Threonine	Aspartic acid	Isoleucine	92	113
10	***Guanine***	Pyridoxine	Tyrosine	Sodium thiosulphate	Proline	Histidine	150	26
11	/	Thiamine	P-amino benzoic acid	Methionine	Glutamic acid	Lysine	100	105
12	Uracil	Riboflavin	Nicotinic acid	Choline	Inositol	Valine	7	0
OD_{625} % of control	202	100	100	100	104	117		
Plasmid % of control	23	146	153	141	124	110		

Crossover nutrients are shown in bold italics

Figure 1. Effect of casein hydrolysate concentration on final biomass and specific plasmid yield for cultures of *E. coli* DH5α pSVβ grown in McCartney bottles on DM medium, supplemented as indicated. ■ biomass (OD_{625}); □ specific plasmid yield ($mg/L/OD_{625}$). Values shown are the means of triplicate determinations, error bars represent 1 standard deviation.

Figure 2. OUR profile for *E. coli* DH5α pSVβ grown in batch culture. The culture was grown on SDCAS in a 7 L fermenter with a 5.5 L working volume, as described in Materials and Methods.

Figure 3. (A) Biomass and plasmid yield, (B) % plasmid supercoiling and (C) nutrient profiles for *E. coli* DH5α pSVβ grown in batch culture. The culture was grown on SDCAS in a 7 L fermenter with a 5.5 L working volume, as described in Materials and Methods. (Δ) DCW, (●) volumetric plasmid yield, (○) specific plasmid yield, (X) D-glucose, (◊) total amino acids.

Figure 4. OUR profile for *E. coli* DH5α pSVβ grown in fed-batch culture. The culture was grown on SDCAS in a 7 L fermenter with a 5.5 L working volume, as described in Materials and Methods. The dashed lines represent the commencement of an exponential D-glucose feed to achieve $\mu = 0.1\ h^{-1}$. The dotted lines represent the commencement of a linear amino acids feed.

Figure 5. (A) Biomass and plasmid yield, (B) % plasmid supercoiling and (C) nutrient profiles for *E. coli* DH5α pSVβ grown in fed-batch culture. The culture was grown on SDCAS in a 7 L fermenter with a 5.5 L working volume, as described in Materials and Methods. (Δ) DCW, (●) volumetric plasmid yield, (○) specific plasmid yield, (X) D-glucose, (◊) total amino acids. The dashed lines represent the commencement of an exponential D-glucose feed to achieve $\mu = 0.1\ h^{-1}$. The dotted lines represent the commencement of a linear amino acids feed.

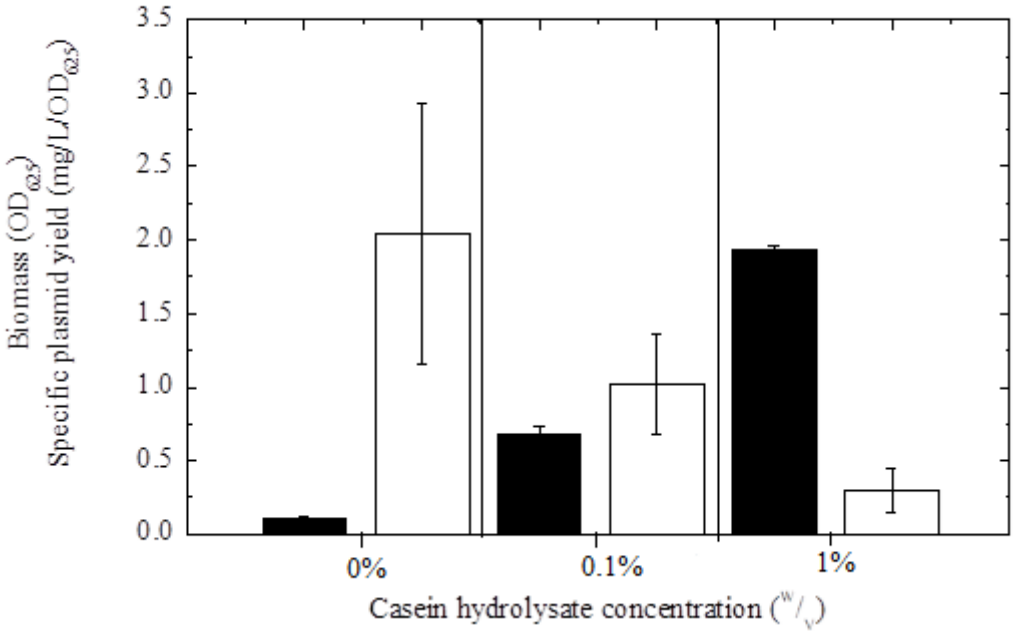
Biomass (OD_{625})
Specific plasmid yield (mg/L/OD_{625})
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0%
0.1%
1%
Casein hydrolysate concentration (w/v)

Figure 1

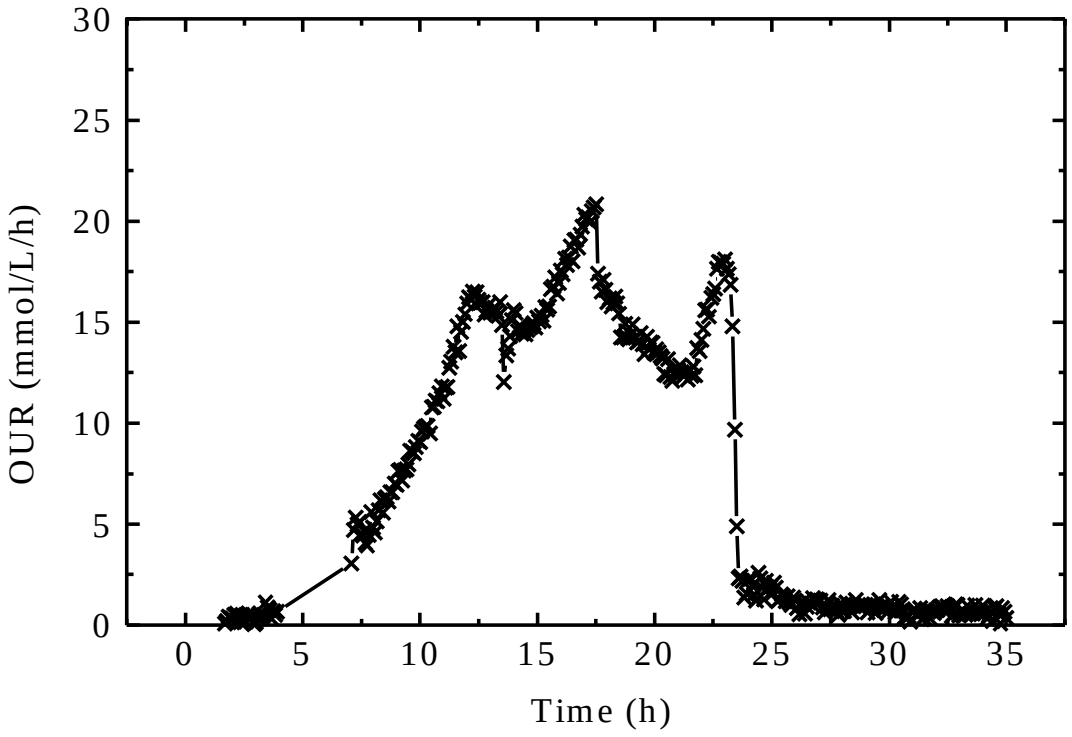

Figure 2

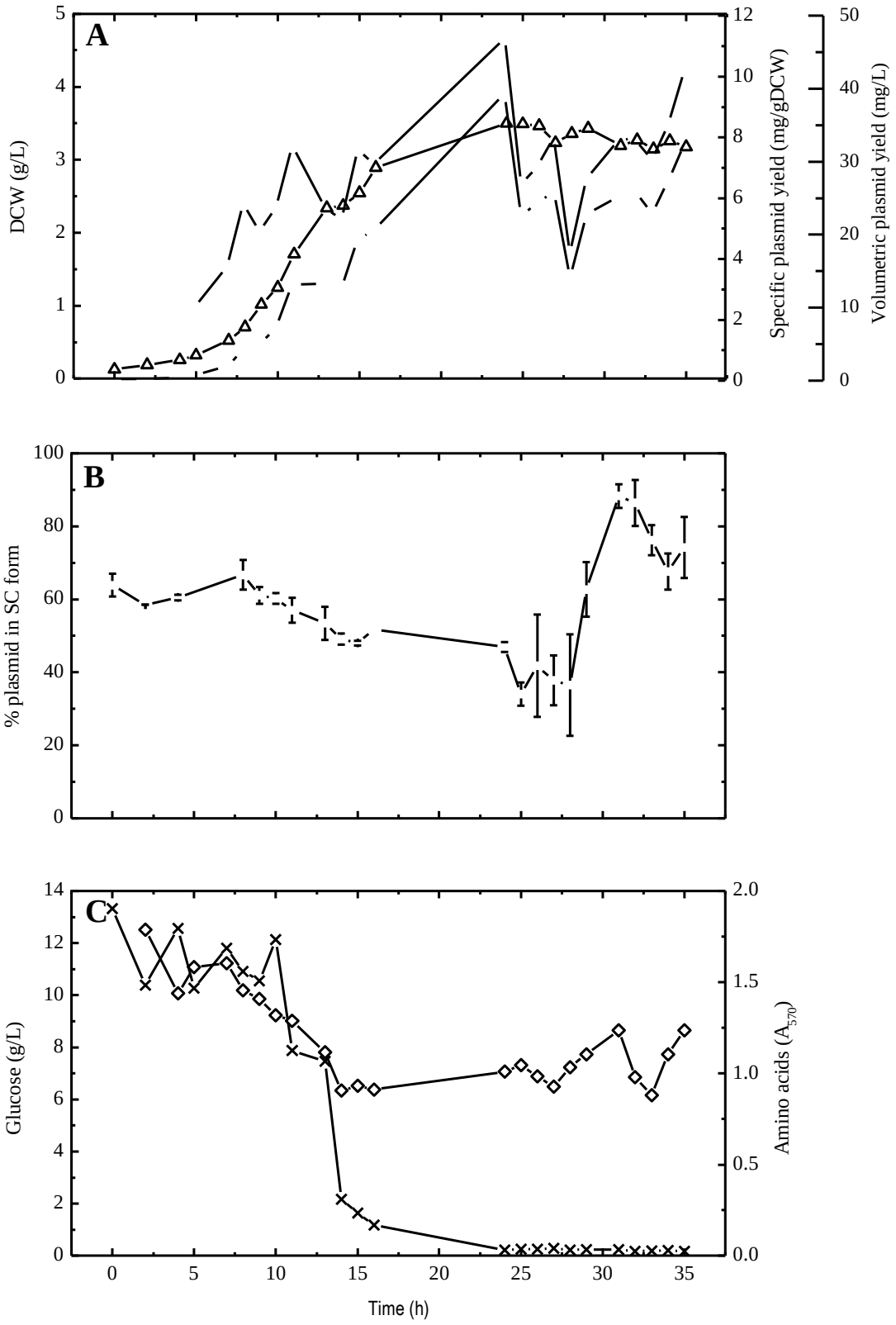
A
DCW (g/L)
Specific plasmid yield (mg/gDCW)
Volumetric plasmid yield (mg/L)
B
% plasmid in SC form
C
Glucose (g/L)
Amino acids (A_{570})
Time (h)

Figure 3

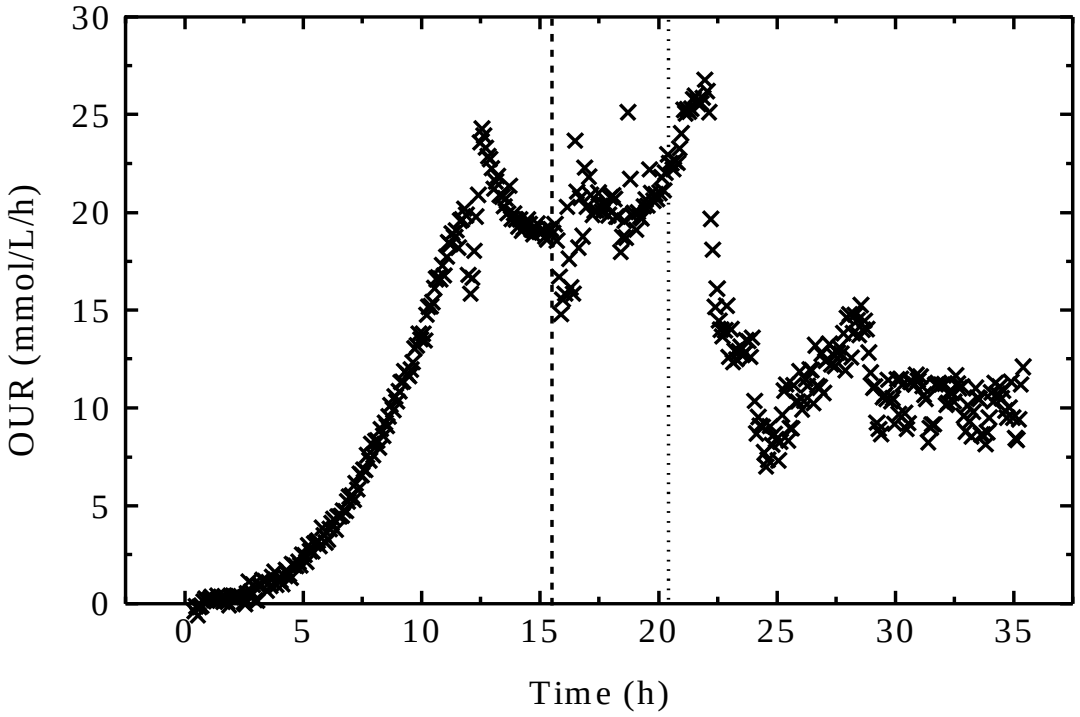

Figure 4

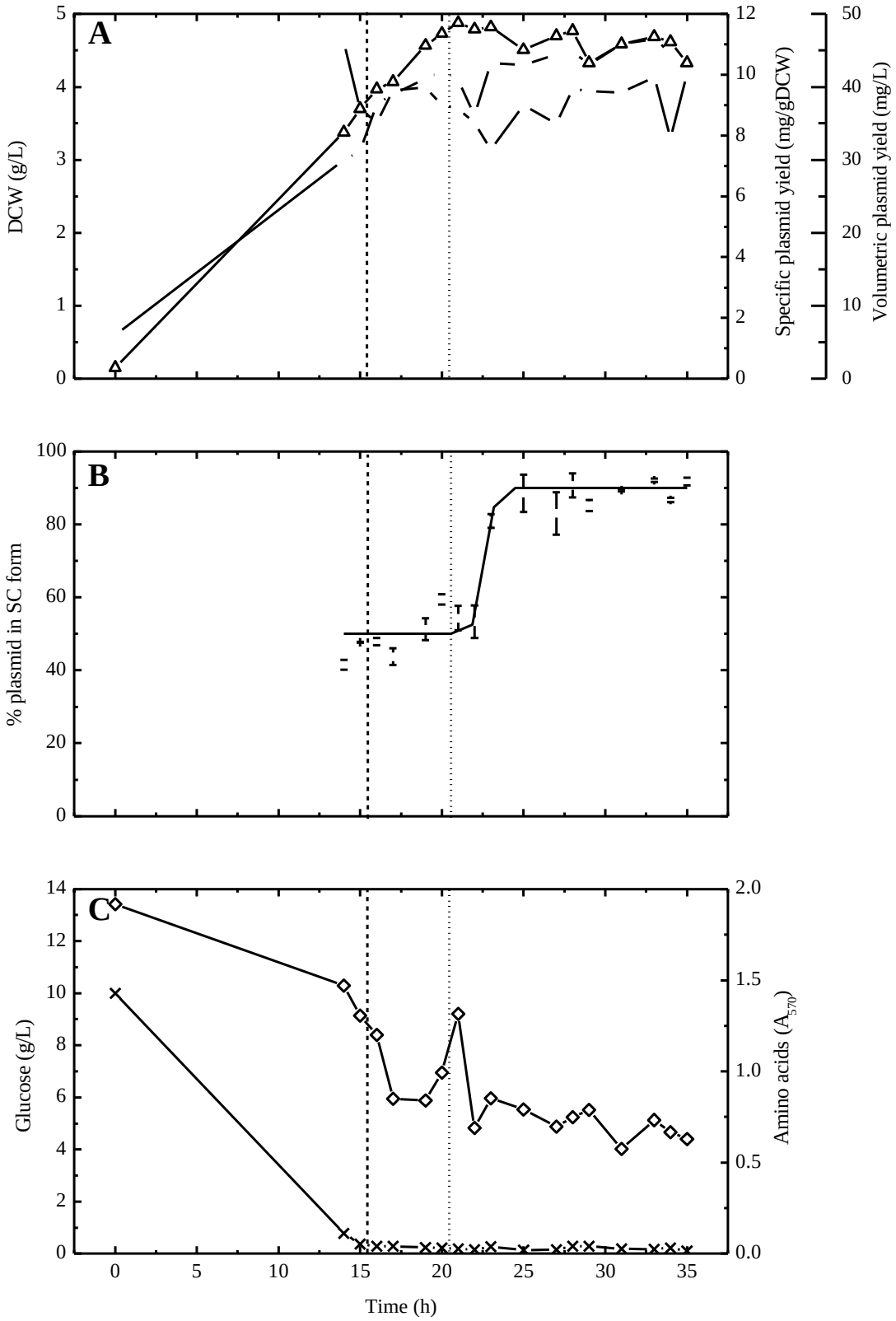
A
DCW (g/L)
Specific plasmid yield (mg/gDCW)
Volumetric plasmid yield (mg/L)
B
% plasmid in SC form
C
Glucose (g/L)
Amino acids (A_{570})
Time (h)

Figure 5