Flow Cytometry and Phytochemical Analysis of a Sunflower Cell Suspension Culture in a 5-L Bioreactor[§]

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A cell suspension culture of sunflower (*Helianthus annuus*), a producer of immunologically active polysaccharides, was cultivated in a 5-L stirred tank bioreactor, operated in batch mode. After some changes in the internal bioreactor design a stable growth of *Helianthus* cells was achieved and the accumulated biomass reached 15.2 g/L (only ~5% lower compared to the accumulated biomass in shake-flasks). Flow cytometry used for measuring the cell cycle parameters of suspended *Helianthus* cells did not reveal significant differences between shake-flasks and bioreactor cultivation modes. For both cultivation methods significant enhancement of the percentage of S-phase cells was observed at the beginning of the cultivation process. Concerning the metabolite production the maximum in exopolysaccharides was reached at day 9 of the cultivation period (1.9 g/L), while the highest amounts of α-tocopherol were accumulated at the beginning of the cultivation process (day 2 of the cultivation). These finding were related to the respective stress levels caused by the inoculation procedure. The kinetic parameters of growth and polysaccharide production as well as the time course of carbon source utilization were monitored and discussed.

Key words: Cell Cycle, Exopolysaccharides, *Helianthus annuus*, Tocopherol

Introduction

Plant cell cultures are considered as an attractive alternative to the classical technologies for the production of bioactive compounds (Rao and Ravishankar, 2002). Furthermore, plant *in vitro* systems appear to be the only source for the production of high value naturally occurring metabolites from rare and threatened plants, which contribute to the global biodiversity conservation (Georgiev *et al.*, 2007). To date large-scale processes for mass production of metabolites have been developed mainly using dispersed plant cell suspensions (Yanpaisan *et al.*, 1998; Kieran, 2001). Cell suspension cultures possess several advantages over other plant *in vitro* systems, for example their resemblance to microbial producers, which allow utilization of well-known bioreactor designs (in some cases with slight modification), easy aseptic inoculation, and higher growth rates. A disadvantage is that plant cells in suspension cultures are very heterogeneous and genetically unstable (Zhong, 2001). From an engineering perspective, the successful application of plant cell suspensions requires large-scale processing technology which supports high-density cultivation of high-yielding, stable cell lines (Kieran, 2001). Cell line selection is frequently used as a powerful tool for both establishment of homogeneous lines and enhancement of metabolite accumulation (Georgiev *et al.*, 2006). Another major point is the development of on-/off-line methods for the determination of cell growth and the physiological behaviour of the cells during their cultivation in different systems. Recently flow cytometry became a popular method for ploidy screening, detection of mixoploidy and

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aneuploidy, cell cycle analysis, estimation of absolute DNA amount or genome size (Yanpaisan *et al.*, 1998, 1999) and thus could be used for biomonitoring.

A cell suspension culture of sunflower (*Helianthus annuus*) was found to produce extracellular polysaccharides (exopolysaccharides) with immunostimulating properties. They induce migration of peritoneal exudative cells (possessing high bactericidic activity), especially peritoneal macrophages, into the peritoneal cavity of experimental animals (Kratchanova *et al.*, 1996). In a previous study a cell suspension culture of *Helianthus annuus* was cultivated in shake-flasks, and based on the achieved growth behaviour and metabolite activity the cell producer was evaluated as promising (Pavlov *et al.*, 2005) and therefore could be used as a good model system. Thus, the present study was conducted to investigate the further bioreactor cultivation with respect to both growth of the cells and metabolites accumulation/production (exopolysaccharides and α-tocopherol). Flow cytometry was used to study the physiological properties of suspended *Helianthus* cells cultivated in a stirred bioreactor vessel.

Materials and Methods

Helianthus annuus cell culture

A callus culture from *Helianthus annuus* was provided by courtesy of Prof. Dr. M. Ilieva (Laboratory of Applied Microbiology and Biotechnology, Institute of Microbiology, Bulgarian Academy of Sciences, Plovdiv, Bulgaria). The callus culture was maintained on Linsmayer and Skoog (LS) medium (Duchefa, Haarlem, The Netherlands, Cat. No. L0230), supplemented with 30 g/L sucrose, 0.2 mg/L 2,4-dichlorophenoxyacetic acid and 5.55 g/L Phytoagar (Duchefa, Cat. Nos. S0809, D0911, P1003, respectively), during a period of subcultivation of 21 d. The suspension culture was grown and supported in the above described medium without Phytoagar and subcultivated every 8 d.

Bioreactor experimental scheme

Experiments were performed in a 5-L stirred tank bioreactor (Bioflo III, New Brunswick Scientific, Edison, USA) equipped with two Rushton turbine impellers. For each cultivation, the working volume of the bioreactor was 3 L. LS medium was filled in the reactor and sterilized at 121 °C for 30 min. After cooling to 26 °C the medium was inoculated with 20% (v/v) 8-day-old cell suspension. Agitation speed was fixed at 100 rpm and the air flow rate was consecutively 0.5 L/min compressed air (days 0–2) and 1 L/min (days 2–11). Control cultivations were performed in 250-mL Erlenmeyer flasks (50 mL net volume) on a shaker at 110 rpm, in the dark, at 26 °C.

Cell growth, conductivity and pH value

The growth of the cells was monitored through determination of fresh and dry biomass (gravimetrically at 60 °C for ~24 h). The kinetic parameters were determined as follows:

$$\partial X / \partial t = \mu_{\max} \cdot X, \tag{1}$$

$$\ln X / X_0 = \mu_{\max} \cdot \Delta t, \tag{2}$$

$$\ln X = \mu_{\max} \cdot \Delta t + \ln X_0, \tag{3}$$

where X_0 and X are the initial and final cell biomass (g), Δt is the culture time (d) and $\mu_{\max}$ is the maximal growth rate (1/d).

Following equation (3) the maximal growth rate was determined graphically. The minimal doubling time (t_d) was calculated as follows:

$$t_d = \ln 2 / \mu_{\max}. \tag{4}$$

The conductivity and the pH value of the culture medium were determined using conductivity- (Qcond 2400 Conductivity Meter Set, VWR International, Darmstadt, Germany) and pH-meters (InnoLab pH720, WTW, Weilheim, Germany).

Sucrose, glucose and fructose determination

Sucrose, glucose and fructose levels of the culture medium were determined in one batch using an enzyme test kit (R-Pharm, Germany, Cat. No. 10716260035).

Flow cytometry and cell cycle

A flow cytometer CyFlow® SL blue (Partec, Münster, Germany) with a 488 nm solid state laser (20 mW) was used. In order to extract the nuclei from the *H. annuus* cells ~0.53 g wet biomass was chopped in a Petri dish with a scalpel for ~6 min in 1.5 mL Marie's nuclear isolation buffer (Marie and Brown, 1993). The staining occurred by addition of 80 μL propidium iodide solution (1 mg/mL; Fluka, Buchs, Switzerland). After 15 min of incubation at room temperature in the dark the liquid was filtered through a 31 μm filter tissue (Poly-

amid 6.6 monofil, Schwegmann Filtrations-Technik, Grafschaft-Gelsdorf, Germany). The fluorescence was detected through a high-pass filter with a cut-off wavelength of 630 nm (FL3) and the trigger was set to FL3. For each measurement at least 10,000 nuclei were counted.

Determination of α-tocopherol

The extraction and derivatization method was according to Franke *et al.* (2007) with slight modifications. Approx. 5 g frozen biomass were mixed with some granules of 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT; Sigma-Aldrich, Steinheim, Germany) as an antioxidant and both were grounded in a mortar. The grinding was continued after adding about 10 mL *n*-hexane (for HPLC; VWR International BDH Prolabo, Leicestershire, England) and then the mixture was centrifuged at $5{,}000 \times g$ for 10 min. The upper hexane phase was transferred to a glass test tube with a Pasteur pipette and dried over anhydrous Na_2SO_4. The obtained extract was evaporated to dryness (rotary vacuum evaporator) and the formed oily residue was resuspended two times with 0.5 mL hexane and placed in an Eppendorf tube. After a centrifugation step for 5 min at $13{,}000 \times g$ the supernatant was removed carefully and transferred into a glass vial for derivatization. Again the solution was evaporated to dryness under N_2, then dissolved in 80 μL pyridine (Sigma-Aldrich). To this solution 80 μL *N*-methyl-*N*-(trimethylsilyl)trifluoroacetic acid (MSTFA; Sigma-Aldrich) for the silylation were added and the mixture was incubated for 30 min at 37 °C, followed by 2 h at room temperature. Subsequently the sample was evaporated to dryness and resuspended in 50 μL ethylacetate (for analysis; Riedel de Haën, Seelze, Germany). The sample was transferred into a glass vial for measurement (injection volume was 2 μL). The analysis was performed on a GC-MS Saturn 2100 instrument (Varian, Palo Alto, CA, USA) equipped with a Zebron ZB-5 (30 m × 0.25 mm × 0.25 μm; Phenomenex, Aschaffenburg, Germany) GC column. As carrier gas helium was used at a flow rate of 1 mL/min. Injector temperature was 250 °C and the temperature program was as follows: 150 °C for 2 min, then with a heating rate of 25 °C/min to 300 °C and at 300 °C for 10 min. The retention time of α-tocopherol was 10.95 min. Electron-impact ionization was realized at 70 eV and fragments were detected with an ion-trap mass analyzer. Identification and calculation of endogenous α-tocopherol were based on the trace of ion $m/z = 503$.

Determination of exopolysaccharides

The exopolysaccharides (EPS) content was determined following the procedure described by Pavlov *et al.* (2005). The product formation rate (r_{eps}) and specific product formation rate (q_{eps}) were calculated according to:

$$\Delta EPS/\Delta t = r_{eps} = q_{eps} \cdot X_{mean}, \quad (5)$$

$$X_{mean} = \Delta X/2, \quad (6)$$

and the product yield ($Y_{eps/x}$) according to:

$$Y_{eps/x} = (EPS_{t=0\ d} - EPS_{t=9\ d})/(X_{t=0\ d} - X_{t=9\ d}) = r_{eps}/r_x. \quad (7)$$

Statistical analysis of the data

The data presented are averages from at least three independent experiments. All measurements were repeated in triplicate.

Results and Discussion

The cultivation of plant *in vitro* systems in bioreactors represents a critical step in the development and scale-up of technologies for the production of target metabolites. This up-scaling is not always easily accomplished with respect to keep the biosynthetic potential of plant cell suspension cultures (Godoy-Hernandez *et al.*, 2000). The main difficulties appear from the fact that the biosynthesis of desired metabolites is influenced by several factors with different significance to the process, and depends on the cultivation method and physiological peculiarities of the cell culture. In previous experiments it was revealed that *Helianthus annuus* cell suspension cultures possess stable growth and good exopolysaccharide biosynthetic characteristics (Pavlov *et al.*, 2005). It was also found that during the cultivation in a stirred tank reactor a significant flotation of the cells appeared, which finally hindered the up-scaling process (Werner, 2005). Through several changes in the internal bioreactor design [introduction of a covering silicone tube with number of fine holes for improved aeration and replacement of the propeller impeller by two Rushton turbine impellers (New Brunswick Scientific, Edison, USA)] the flotation was prevented and the system was adjusted for the cultivation of *Helianthus* cells.

Growth and physiological characteristics in the bioreactor

The time course of growth of the *Helianthus* cells and the medium conductivity changes during the cultivation in the stirred tank reactor were studied (Fig. 1A). It was observed that the culture grew intensively and the maximum of biomass accumulation was reached at the 10th day of cultivation, reaching 15.2 g/L (only ~5% lower compared to the accumulated biomass in shake-flasks stage). The maximal growth rate (μ_{max} = 0.2 1/d) was ascertained between days 1–7 and the minimal doubling time was ~83 h. Although the calculated kinetic parameters were lower compared to those reported earlier for *Helianthus annuus* cell suspension cultures, the biomass productivity (1.5 g/L/d) was significantly higher even when an air-lift bioreactor system was used (Scragg, 1990). The conductivity of the medium decreased during the course of the cultivation (Fig. 1A), which is presumably due to the uptake of ionic compounds from the culture medium. It has been shown that the increase in cell concentration could be correlated directly with the decrease in medium conductivity (Ryu and Lee, 1990; Suresh *et al.*, 2001). Such linear interdependence with a high statistical significance (r^2 = 0.99) was developed for *Helianthus* cells (Fig. 1D), which could be applied for further fast (compared to the widely used methods) indirect monitoring of cell growth for a better control of biosynthetic processes during the scale-up.

To further characterize the behaviour of the *Helianthus* cells in the stirred tank reactor, the time course of the utilization of the carbon source was monitored (Fig. 1B). The sucrose consumption began with a rapid hydrolysis in the culture medium, catalyzed by the cell wall invertase (Shin *et al.*, 2003), being completely exhausted at day 8 of cultivation. In shake-flasks culture some residues could be detected until day 10 (Pavlov *et al.*, 2005), which indicate higher invertase activity in bioreactor cultures. The two monosaccharides glucose and fructose were metabolized in similar temporal patterns being completely utilized at the end of the cultivation process (Fig. 1C).

The time course changes of dissolved oxygen (DO2) were also followed during the cultivation in the stirred tank reactor (Fig. 1C). At the beginning of the cultivation process dissolved oxygen level slightly decreased until day 2 followed by an increase, which was due to the enhancement of the air flow rate (from 0.5 L/min to 1.0 L/min). Between days 2–9 the dissolved oxygen level significantly decreased which coincided with the intensive cells growth causing intensive respiration (Fig. 1C). The beginning of the oscillation of the

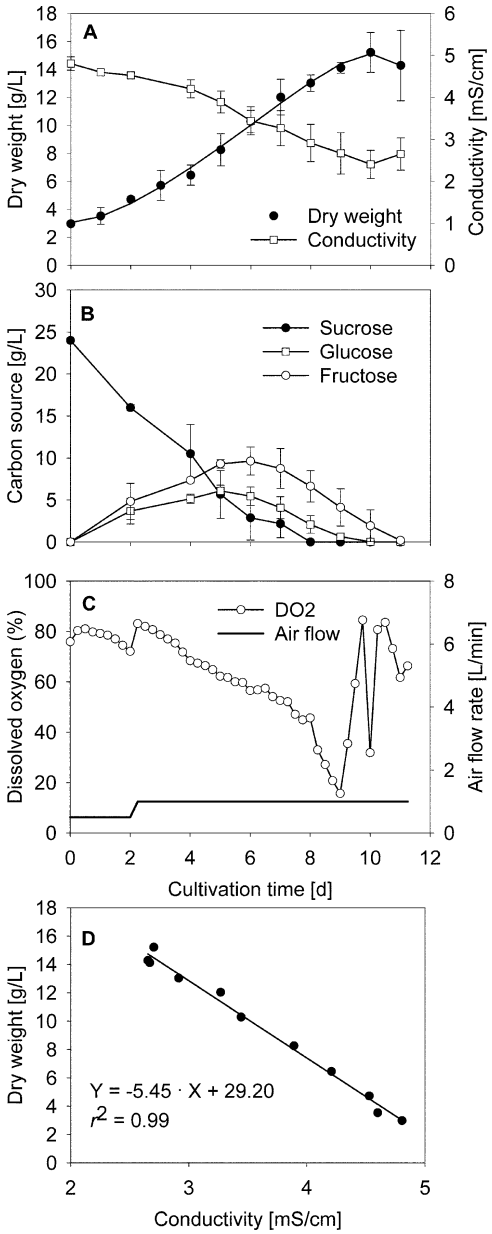

Fig. 1. Time courses of (A) growth and conductivity changes, (B) carbon source utilization, (C) dissolved oxygen and air flow rate changes, and (D) linear dependence between dry weight and conductivity during the cultivation of the *Helianthus annuus* cell suspension culture in a 5-L stirred tank reactor.

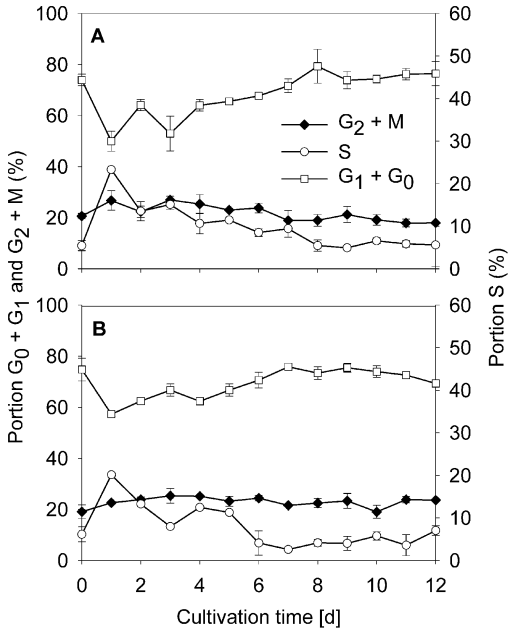

Fig. 2. Portion of the cell population of (G_0 + G_1)-, S- and (G_2 + M)-phases during the cultivation of the *Helianthus annuus* cell suspension culture (A) in shake-flasks and (B) in a 5-L stirred tank reactor.

dissolved oxygen level at day 9 was due to the enhanced culture medium viscosity, which hampered the oxygen mass transfer and exact measurement of the dissolved oxygen level (Schuegerl, 1981).

Cell cycle analyses

Flow cytometry was used to measure cell cycle parameters in the *Helianthus* suspension culture (Fig. 2). After the transfer to new medium cell proliferation sets on, the G_1 stage shortened and cells from G_1 enter consecutively to S- and G_2/M-phases. As a consequence the fraction of G_0/G_1 decreases, which corresponds to the increase of the portions of S and G_2/M. The high percentage of G_2/M- and S-phase cells was in agreement with the results from culture growth (dry weight curve), where after inoculation there was a period of exponential growth. The highest percentage of G_2/M-phase cells (27.0%) was observed on the 3rd day. Subsequently this portion slightly decreased, but never fell below 18%. The percentage of S-phase cells decreased to their start level at day 8 and then remained nearly constant. The G_0/G_1-cells had the lowest portion in shake-flasks at the 1st day with 50% and increased from the 3rd day continuously, amounting to ~70% at day 7 (Fig. 2A). Similar results were reported by Yanpaisan *et al.* (1998) for the cultivation of cell suspension cultures of *Solanum aviculare* in shake-flasks.

The bioreactor cultivation showed a similar pattern with rather small differences in the cycle phases in the course of the cultivation (Fig. 2B). The biggest differences again could be found shortly after the inoculation. Despite the similar pattern in the time course slight changes were observed – the maximal G_2/M-value was always below the one in shake-flasks (25.3% vs. 27.0%). Consequently, the minimal G_0/G_1-value of the bioreactor culture was higher than that for the shake-flasks, which indicates that the cells grow faster in the shake-flasks than in the bioreactor. This was in good agreement with the maximal specific growth rates determined for the corresponding cultivation systems (0.26 vs. 0.20 1/d, respectively) and gave an indication that the G_0/G_1-level can be used as a marker for the growth of cells during the cultivation.

If all cells would finish the cell cycle, it could be expected that the amount of cells in the G_2/M-phase would approach to zero at the end of the batch cultivation. However, at the end of the cultivation, we observed a fraction of ~18–24% G_2/M-phase cells (shake-flasks and bioreactor). This phenomenon could be due to: 1) the existence of quiescent *H. annuus* G_2-cells (Sgorbati *et al.*, 1989) and/or 2) a slight tendency of the cells to undergo endoreduplication (genome duplication without mitosis). Endoreduplication is widespread in plants, particularly in angiosperms, and may occur in any cell type except for the gametes, the meristematic and guard cells. Polysomatic cells do not proliferate and hence do not participate in the cell cycle (Cebolla *et al.*, 1999). In this case the cells that underwent one cycle of endoreduplication possess the same DNA amount as the (G_2 + M)-phase cells of the cycling subpopulation. Furthermore it can be assumed that quiescent cells exist in the (G_0 + G_1)-fraction as this fraction never falls below ca. 50%. It should also be noted that the one parameter analysis of DNA content does not provide enough information to distinct cycling form quiescent cells, so further experiments will be focused on biparametric cell cycle analysis.

Metabolite accumulation and production

The capacity of a *Helianthus annuus* cell suspension to produce metabolites was investigated through determination of intracellular (vitamin E) and extracellular (exopolysaccharides) compounds. In a previous study Kratchanova *et al.* (1996) found that the *Helianthus* EPS possess high immunostimulating activity. It was also determined that the optimal inoculum amount is 20% (v/v). In a more recent study Pavlov *et al.* (2005) investigated the kinetics of EPS production in shake-flasks stage and outlined the high potential of a *Helianthus annuus* cell suspension culture for their synthesis. Thus, the time course of EPS production by *Helianthus* cells was investigated during the cultivation in a 5-L stirred tank reactor (Fig. 3A). It was observed that the active EPS production started at day 4 of the cultivation showing a maximum at day 9 (~1.9 g/L). The product formation rate (r_{eps}) and the specific product formation rate (q_{eps}) as well as the product yield related to biomass ($Y_{eps/x}$) were determined for the interval of maximal production and are presented in Fig. 3A. The comparison with shake-flasks cultivation showed that all parameters calculated were ~2-fold lower at the bioreactor stage. Surprisingly for us the levels of some stress-related hormones (jasmonic acid and abscisic acid) were found to be lower in the bioreactor culture than in the shake-flasks one (data not shown) indicating a higher stress situation in flasks, which probably influences the production of EPS as a part of the cell defense system. All of this implicates that the internal bioreactor environment (*e.g.* air flow rate, agitation speed, temperature) should be optimized to achieve improved high values of EPS production.

α-Tocopherol (vitamin E), a compound with high value biological activities, was determined in cultured *Helianthus* cells by GC-MS. Because of the large diversity of substances present in the *Helianthus* sample it was difficult to find an appropriate internal standard for measurements which is not present as natural compound. Therefore we splitted the samples and added a defined amount of α-tocopherol to one part and then, by comparing the peak areas of samples with added α-tocopherol and unmodified samples, the α-tocopherol levels were determined. The values for day 0 were set to 100% and the other values were normalized to this basis (Fig. 3B). Significant enhancement of the α-tocopherol content was observed at the beginning of the cultivation process (~3-fold in shake-flasks culture and 2.6-fold in bioreactor culture). This enhancement could be explained with the higher stress levels, caused by the inoculation procedure and the role of tocopherols as antioxidants. Munne-Bosch and Alegre (2002) proposed that α-tocopherol may affect intracellular signaling in plant cells either direct, by interacting with key components of the signaling cascade, or indirect, through the prevention of lipid peroxidation and/or the scavenging of singlet oxygen. Furthermore this supports the statement that the yields of α-tocopherol could be increased by subjecting the cells to different stress factors (Gala *et al.*, 2005). From day 2 of cultivation a decreasing trend was observed, probably indicating the decreased stress levels. It should be also noted that the α-tocopherol content in the bioreactor culture was almost always higher compared to shake-flasks, which is due to the higher aeration rates and thus the enhanced production of reactive oxygen species.

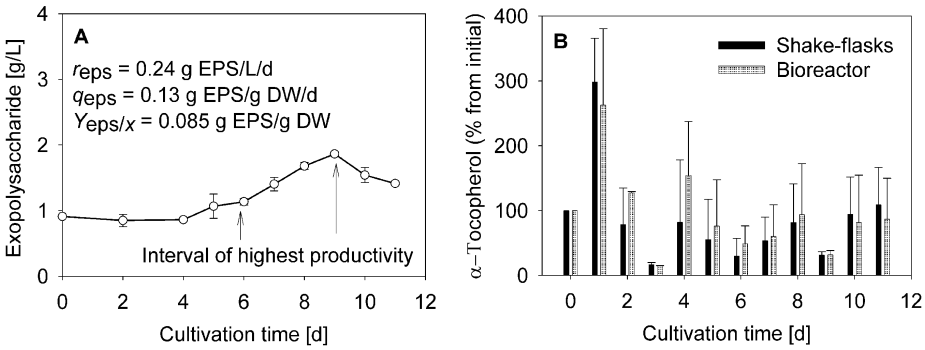

Fig. 3. Time courses of (A) exopolysaccharide production and (B) α-tocopherol accumulation during the cultivation of *Helianthus annuus* cell suspension culture.

In conclusion, the plant cell suspension culture of sunflower, cultured in stirred tank bioreactor showed stable growth and accumulated high biomass amounts. The cell cycle analysis did not reveal significant changes between bioreactor and shake-flasks cultivation. The production of high value metabolites was proven and the necessity of amounts of optimization of the internal bioreactor environment was outlined.

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