A Simplified Strategy for the Release and Primary Recovery of c-phycocyanin Produced by *Spirulina maxima*

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A simplified strategy for the potential production, release and primary recovery of c-phycocyanin from *Spirulina maxima* in one single unit operation, is discussed. The strategy exploited the use of a bioreactor loaded with solids (e.g. glass beads) and operated under define conditions as an attractive alternative to mimic a high speed bead mill. Selected operating quantities (e.g. glass beads diameter and loading, speed of agitation, $CaCl_2$, and biomass concentration) were evaluated to define the appropriate conditions for cell disruption. Once fermentation is terminated cell disruption was performed after broth conditioning, primary recovery of the protein was achieved simultaneously, using aqueous two-phase extraction. The addition of the chemical forming phases (phosphate and poly ethylene glycol (PEG)) directly to the fermentation device allowed the *in situ* recovery of c-phycocyanin. The operating conditions established for the cell disruption step (i.e. glass beads of 0.8 – 1.0 mm diameter, speed agitation of 1,500 rpm, beads loading of l = 38 %, 10 g L^{-1} $CaCl_2$ and a 12 g L^{-1} biomass concentration), together with those of the ATPS extraction (i.e. w = 7 % of PEG 1450 and w = 20 % phosphate), resulted in one-stage process for the potential recovery of c-phycocyanin (with a purity of 1.7, defined as the relation of 620 nm to 280 nm absorbances) from *Spirulina maxima*. Such primary recovery step can be further used for the development of a prototype purification process as a first step for the commercial production of c-phycocyanin produced by *Spirulina maxima*.

Key words:
Bioseparation, cell disruption, protein recovery, c-phycocyanin, *Spirulina maxima*

Introduction

The increasing need to rapidly and economically bring new biopharmaceutical products to market using scaleable and efficient technology has encouraged manufacturers to seek competitive advantage through bioprocess intensification. With the increasing commercial significance of colouring compounds used in food, cosmetic, detergent and molecular genetics industries[1, 2] there is a need to bring these products to market using biotechnological processes. In this context, the production of c-phycocyanin (a blue coloured protein) by *Spirulina maxima* represents a very interesting case because, both, the industrial application and commercial value of this product are considerable[1]. The commercial value of food grade c-phycocyanin (purity of 0.7, defined as the relation of 620 nm to 280 nm absorbances) is around 0.13 U.S.$ mg^{-1}, whilst that of reactive grade c-phycocyanin (purity of 3.9) varies from 1 to 5 U.S.$ mg^{-1}.[3] In contrast, the commercial value of analytical grade c-phycocyanin (purity greater than 4.0) can be as high as 15 U.S.$ mg^{-1}.[4] c-phycocyanin is one of the two main biliproteins from the photosynthetic systems of *Spirulina maxima*. It is formed by two sub-units, α and β, of 20.5 and 23.5 kDa of molecular mass, respectively,[5] and its isoelectric point has been reported[6] to be around 5.8.

In order to recover c-phycocyanin from *Spirulina maxima* a protocol was proposed by *Herrera et al*[3], that involves stages of, harvesting, drying, milling, extraction with salts ($NaNO_3$ and $CaCl_2$), adsorption, ultrafiltration, precipitation, dialysis, gel filtration and chromatography of ion exchange. This procedure resulted in c-phycocyanin of two types: food grade (purity of 0.74) and reactive grade (purity of 3.9). However, this protocol has disadvantages primarily in the negative effect of the excessive number of unit operations (i.e. ten unit operations) upon product yield. To overcome such disadvantage attributed to the proposed protocol, we developed a novel prototype process for c-phycocyanin recovery from *Spirulina maxima*[7]. This

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novel process exploited the use of a two-stage extraction aqueous two-phase systems (ATPS), ultrafiltration and precipitation, to reduce the number of unit operation to five and benefit from an increased yield of the protein product. As a result, a recovery process that produced a protein purity of 3.9 and an overall product yield of 29.5 %, was obtained. However, the scale up of the process described[7] raises complications associated to the stage used for cell disruption. In this case, product release was achieved using milling by hand, which resulted in process problems that limited the commercial application of this prototype process. Although, the use of a mechanical cell disruption device such as bead mill[8] can be implemented in the described process, alternative approaches in the light of bioprocess integration and intensification need to be explored.

The simplified approach of using a bioreactor loaded with solids (e.g. glass beads), and operated under defined conditions as an attractive alternative, to mimic a high speed bead mill for the recovery of c-phycocyanin produced by *Spirulina maxima*, was suggested for the present research. This bioengineering strategy opens the opportunity to potentially perform the production of c-phycocyanin, the release and the primary recovery with ATPS in one single device. Once the fermentation has been terminated, the process conditions can be altered to promote both: *in situ* cell breakage and subsequently primary recovery with ATPS. However, no reports on the release of c-phycocyanin from *Spirulina maxima* in high speed bioreactor loaded with glass beads have been published. The research presented here aims to generate knowledge on the release behaviour of c-phycocyanin from *Spirulina maxima* in a bioreactor tank loaded with glass bead and operated at high speed. A simplified strategy was exploited to potentially obtain the product of interest, release it and extract it in one single unit operation. Such approach resulted in the definition of the primary recovery step for the development of a downstream process for the purification of c-phycocyanin produced by *Spirulina maxima* with potential commercial application.

Materials and methods

Cultivation conditions and cell disruption

Spirulina maxima was cultivated in the culture medium described by *Herrera et al*.[3] The algae were grown in a batch culture (500 ml Erlenmeyer flasks) at 20 °C under natural light conditions, agitation was provided using a reciprocal shaker (LAB-LINE at ω = 80 min^{-1}). The cells were allowed to grow for 12 days until an OD_{560} of 0.7 was reached and were harvested by centrifugation at 12,000 rpm for 10 min (Eppendorf 5415C). After cell harvesting, cellular disruption was performed in an in-house assembled device comprised of a 500 ml flask with a turbine type agitator (3.8 cm of diameter), and loaded with glass beads at different operating conditions (see next section). Temperature was monitored throughout the process and controlled with a dry ice bath when necessary. Complete cellular fragmentation was verified using an optical microscope (Olympus CK2). In the case when complete cellular fragmentation was not achieved, the fraction of cell disruption (%) was estimated relative to the unbroken cells observed. Cell debris removal was achieved by centrifugation at 12,000 rpm for 10 min (Eppendorf 5415C) and the supernatant (referred to as crude extract) was used for analytical purposes.

Establishment of the operating conditions for the release of c-phycocyanin from *Spirulina maxima*

Experimental systems used to establish the operating conditions for the cell disruption process were prepared using fresh biomass (1 to 6 g wet mass) from the fermentation and re-suspended in 500 ml of fermentation broth or a solution contained $CaCl_2$ (10 to 20 g L^{-1}). In order to select the appropriate glass beads, different diameters of beads (0.8 to 5 mm) were evaluated. Loading volume fraction of the glass beads varied from 12 to 38 % (relative to the total volume of the tank, 500 ml) and three (800, 1000 and 1,500 rpm) speed of agitation of the system were also evaluated. During the cell disruption process, samples were carefully taken at certain intervals of time for analysis and subsequent estimation of the level of disruption and the protein content and purity.

Cell disruption and primary recovery with aqueous two-phase systems

Once the optimal conditions for the cell disruption step were obtained, an *in situ* cell disruption and primary recovery with aqueous two-phase systems (ATPS) step was performed. Conditions for the ATPS extraction were taken from a previous report.[7] Predetermined quantities of solid PEG 1450 and potassium phosphate were mixed with the re-suspended biomass of *Spirulina maxima* (12 g L^{-1} in a 10 g L^{-1} $CaCl_2$ solution) to give a final composition of w = 7 % and w = 20 % of PEG and phosphate, respectively. The pH was adjusted to 6.5 with orthophosphoric acid and the glass beads added to operate the cell disruption system. Samples were taken after the procedure was finished and centrifuged to achieve complete phase separation. Top phase was then used for analysis. Results reported

are the average of three independent experiments and errors were estimated to be a maximum of ±10 % of the mean value.

Analytical procedures

Protein concentration in the samples was estimated by the method of *Bradford*.[9] Samples were diluted in deionised water prior to biochemical analysis. The purity of phycocyanin was determined as the relation of the 620 nm to 280 nm absorbance (i.e purity of phycocyanin = Abs620nm/Abs280nm). Purified c-phycocyanin available in our laboratory (obtained following the protocol described by *Herrera et al*[3]) was used as standard to estimate the concentration of the protein.

Results and discussion

Influence of glass beads loading and $CaCl_2$ concentration upon the purity of c-phycocyanin released from *Spirulina maxima*

The bioprocess intensification of the production of biotechnological products can result in the design of unit operation that allows the processing of an increased amount of feedstock material to obtain the desire product. Furthermore, process integration in biotechnology can also imply, that the goals achieved by two or more discrete unit operations may be obtained by one single unit operation. In the context of bioprocess development, we have reported[7] a prototype process for the recovery of c-phycocyanin from *Spirulina maxima*. The potential commercialisation of the process is limited by the cell disruption step. This stage was performed by a manual method using a ceramic pot and glass beads. In order to overcome this limitation, the use of a fermentation flask that mimics a high speed bead mill for the cell disruption step is evaluated. In addition, this strategy opens the opportunity to perform the production of c-phycocyanin, the release and the primary recovery with ATPS in one single device. As a first step, the establishment of the optimal operating conditions for the release of c-phycocyanin from *Spirulina maxima* in a fermentation flask loaded with glass beads, was attempted. Due to the commercial importance of the protein of interest, in this research the purity of the c-phycocyanin is used to evaluate the performance of the process. Preliminary cell disruption experiments (data not shown) demonstrated that the glass beads of 0.8 – 1.0 mm produced the maximal cell disruption (75 %, estimated by observation with an optical microscopy), when the fermentation flask was loaded (up to 20 % of the volume) with glass beads of different diameters. In the same experiments the speed of agitation of 1,500 rpm was selected for the turbine agitator of 3.8 cm of diameter, when different speeds of agitation were tested. In this case the selection of the speed of agitation was based upon the purity of the product released under the conditions studied. Once these operating conditions were selected, the definition of the part of glass beads loading into the system was attempted.

Figure 1 depicted the effect of increasing glass bead loading to the system upon the purity of the c-phycocyanin released. It is clear that an increase in the glass bead loading from 12 % to 38 % (relative to the total volume of the flask) resulted in an increment in the purity from 0.7 to 1.25 (which corresponds to the ratio of the particular release of c-phycocyanin and other intracellular proteins). Similar results for a different biological systems, using a bead mill, have been reported by *Ricci-Silva et al*.[10] In this context, *Chisti* and *Moo-Young*[11] reported that an increase in the loading of the glass beads caused the rate of cell disruption to rise. Such phenomenon was attributed to the fact that the percent of cell disruption is associated to the abrasive forces and the frequency of collision between the glass beads and the cells. Furthermore, it has been reported that the frequency of collision rises exponentially with the increase in the glass bead loading.[12] Such trend can be observed in Figure 1 for the case of the purity of c-phycocyanin released when the loading of glass beads is increased in the cell disruption system. Although, in bead mill a glass bead loading of 85 % is recommended for the release of intracellular products[13, 14] in the present research, and increase of the loading above 38 % was not pursued because after 45 min of operation a cell disruption of 100 % (estimated as the fraction of cells broken observed in a optical microscopy) was obtained.

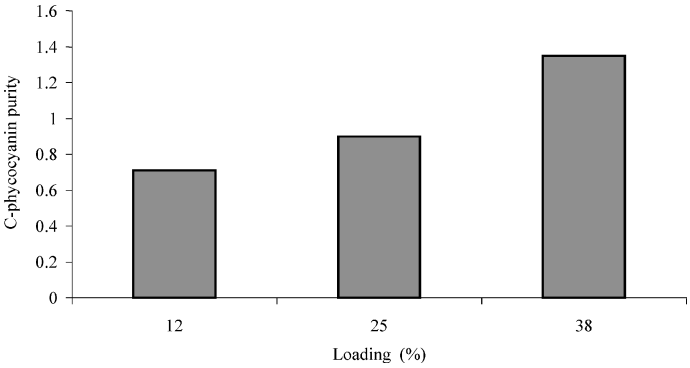

Fig. 1 – *Influence of beads loading upon the purity of c-phycocyanin released from Spirulina maxima. The purity of c-phycocyanin released from the biomass (2 g* L^{-1}*; wet mass) re-suspended in a 10 g* L^{-1} $CaCl_2$ *solution was estimated as the relation of absorbances (Abs620/Abs280) and is expressed relative to the loading fraction. The speed of agitation was 1,500 rpm and the system was operated for 45 min.*

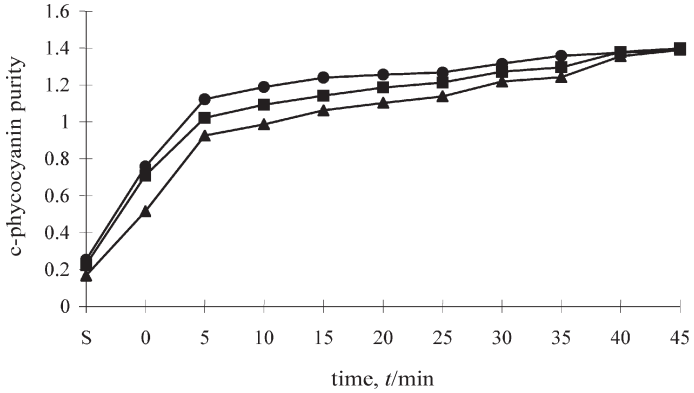

Fig. 2 – *Influence of $CaCl_2$ concentration upon the purity of c-phycocyanin released from Spirulina maxima. The purity of c-phycocyanin released from the biomass re-suspended in 10 g L^{-1} $CaCl_2$ solution (▲), 15 g L^{-1} $CaCl_2$ solution (■) and 20 g L^{-1} $CaCl_2$ solution (●) was estimated as the relation of absorbances (Abs620/Abs280), and is expressed relative to the time of cell disruption.*

The use of $CaCl_2$ as a chemical agent favours the cell disruption, because caused changes in the osmotic pressure[15] and in the case of the c-phycocyanin act as a solvent for the protein[3]. In the present research the effect of the concentration of $CaCl_2$ upon the performance of the cell disruption was evaluated. It is clear that a change in the concentration of the salt from 10 to 20 g L^{-1} in the cell disruption systems caused no significant change in the purity of the protein released (Figure 2). A possible explanation may involve the increase in the solubility of, both, the protein of interest and the contaminants released; as a result the purity of c-phycocyanin remained almost constant for the different salt concentration used. For economic reasons it was decided to use the 10 g L^{-1} concentration of $CaCl_2$ as the appropriate operating condition.

Influence of the fermentation broth and biomass concentration upon the purity of c-phycocyanin released from *Spirulina maxima*

In order to facilitate the integration of fermentation and cell disruption steps, the disruption of the cells was attempted immediately after the fermentation was terminated without prior broth conditioning. By considering the reported results of the previous section, cell disruption was evaluated when $CaCl_2$ was added to the fermentation broth until a 10 g L^{-1} concentration was achieved. As a comparison and to estimate the effect of the nature of the fermentation broth upon the cell disruption performance, disruption was also conducted with re-suspended biomass in a 10 g L^{-1} $CaCl_2$ solution. This latest experiment took no account of the potential impact of components of the fermentation broth (e.g. salts, medium components, etc.) upon the release of the product of interest. Figure 3 illustrates the effect of the fermentation broth components upon the purity of the c-phycocyanin released. The use of the fermentation broth as a medium for the cell disruption step severely affected the performance of the system (the purity of c-phycocyanin was less than 0.4). It was visually observed that, when the $CaCl_2$ was added to the fermentation broth and excessive precipitation of salts was obtained at the end of the cell disruption. It is clear that such situation affected the solubility of the protein (and as a result its purity) in the system. In this context, the findings of *Berns* and *MacColl*[16] concerning, that an increase in the concentration of salts in the medium caused c-phycocyanin to aggregate and precipitate, support the explanation of the effect of the fermentation broth upon the performance of the disruption system. In contrast, the purity of the protein released from the system in which the cells were re-suspended in a 10 g L^{-1} $CaCl_2$ solution was similar to that achieved from the loading experiments. A further information of the samples from the two systems evaluated using an optical microscopy revealed that in both systems the percent of cell breakage was similar. Therefore, it is possible that the effect on the purity of the protein was due to aggregation and precipitation phenomena of the released protein. The results obtained from these experiments imply that a broth conditioning (which can be performed in the same equipment) is required before the cell disruption step.

In order to evaluate the processing capacity of the systems, the effect of an increase in the biomass

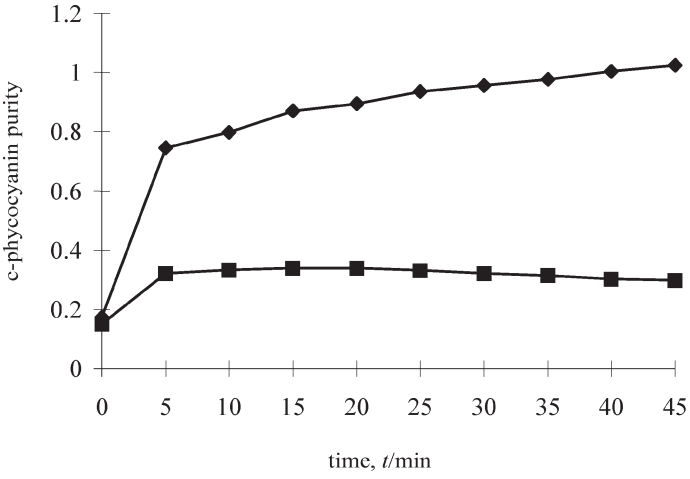

Fig. 3 – *Influence of the fermentation broth upon the purity of c-phycocyanin released from Spirulina maxima. The purity of c-phycocyanin released from the fermentation broth (■) and that from the biomass re-suspended in a 10 g L^{-1} $CaCl_2$ solution (◆) was estimated as the relation of absorbances (Abs620/Abs280) and is expressed relative to the time of cell disruption. The cell disruption was performed using a biomass concentration of 2.0 g L^{-1} (wet mass) and glass beads of 0.8 – 1.0 mm diameter loaded at φ = 38 % of the vessel capacity.*

concentration upon the performance of the cell disruption unit was evaluated. The concentration of the biomass in the disruption system was varied from 2 to 12 g L^{-1}. It has been reported before[10,13,14] that an increase in the cell concentration does not affect significantly the percent release of intracellular products, when a bead mill equipment is used. However, in the case of the release of c-phycocyanin an increase in the biomass concentration resulted in changes in, both, the percent of cell disruption and product yield, whilst the purity remained constant (see Table 1). The purity of c-phycocyanin (1.3 ± 0.1) exhibited no changes, which may be associated to the fact that the ratio of the protein of interest to contaminants released remained constant when the concentration of the biomass increased. On the other hand, it seems that an increase in the biomass concentration (from 2 to 12 g L^{-1}, wet weight/volume) affected the efficiency of system to break the cells (cell disruption fraction decreased from 100 to 85 %); as a result the product yield (expressed as mg of c-phycocyanin/g of biomass, wet mass) decreased. From the results of Table 1, a selection base upon the whole parameters evaluated indicate that the system with 2 g L^{-1} of biomass provided the best conditions for the cell disruption stage. However, such system (containing 2 g L^{-1}) required the use of a diluted biomass (six time compared with that from the system with 12 g L^{-1} biomass concentration) which will result in a excessive repetitive use of the disruption unit to process the biomass. As a result and to avoid a negative view to the process, it was decided that the system comprising a 12g L^{-1} biomass concentration, glass beads of 0.8 – 1.0 mm diameter, speed agitation of 1,500 rpm, loading of φ = 38 % and 10 g L^{-1} $CaCl_2$, provided the best conditions for an intensified process for the release of protein with maximum purity (i.e. 1.3) to evaluate the potential process integration for c-phycocyanin production, release and primary recovery with ATPS from *Spirulina maxima*.

Potential process integration for the release and primary recovery of c-phycocyanin produced by *Spirulina maxima*

Once the conditions for the cell disruption were established the primary recovery with ATPS of c-phycocyanin from *Spirulina maxima* using the disruption system was attempted. In the previous prototype process for c-phycocyanin recovery reported,[7] the purity of the protein was increased up to 2.0 using discrete unit operations of cell disruption and ATPS extraction. In the present research, the cell disruption and ATPS extraction were performed simultaneously in the fermentation flask after the fermentation was terminated (following the operating conditions described in sections 3.1 and 3.2) and the broth conditioned. In this case, once the fermentation was finished, the broth was replaced by a solution of 10 g L^{-1} of $CaCl_2$ (conditioning of the broth). After the biomass was re-suspended in the $CaCl_2$ solution, solid PEG 1450 and phosphate were added until a w = 7 % and 20 % concentration, was achieved respectively. The conditions for the ATPS extraction were taken from the previous report[7] without further modification. The operation of the disruption system was initiated to allow PEG and phosphate solid dissolution and cell disruption. The purity of the c-phycocyanin, obtained from the top PEG-rich phase derived from this system, was 1.7 ± 0.1. The decrease in the purity obtained compared to that reported previously[7] (i.e. 2.0), can be attributed to the performance of the ATPS under the simultaneous conditions of the cell disruption and the extraction stages. Apparently, the presence of the intracellular products and the operating conditions affected the partition behaviour of the protein and contaminants in a manner that can not be currently explained. In this context, similar results were reported by *Rito-Palomares* and *Lyddiatt*[17] for process integration of cell disruption and ATPS for the recovery of intracellular protein from yeast. However, the results reported here facilitate the potential process integration for the production, release, and primary recovery of c-phyco-

Table 1 – *Influence of biomass concentration upon cell disruption of Spirulina maxima for the release of c-phycocyanin*

Biomass concentration γ_{BIO}/g L^{-1}	Cell disruption X %	c-phycocyanin purity	c-phycocyanin yield Y (mg protein/g biomass)
2.0	100.0	1.3 ± 0.1	14.4 ± 1.4
6.0	90.0	1.3 ± 0.1	7.7 ± 0.8
12.0	85.0	1.3 ± 0.1	7.0 ± 0.8

Biomass concentration is expressed as wet mass per volume. The number fraction (%) of cell disruption was estimated by visual observation using an optical microscope (Olympus CK2) as described in Materials and Methods. The purity of c-phycocyanin is expressed as the relation of the 620 nm to 280 nm absorbances. The yield of c-phycocyanin is expressed relative to the initial wet mass of the biomass loaded to the systems.

cyanin from *Spirulina maxima* in one single unit operation. Additionally, the potential fermentation of *Spirulina maxima* in ATPS deserves attention. In this particular case, the effect of chemical forming phases for the ATPS (e.g. polymer and salts) upon the fermentation performance needs to be addressed.

It is clear that economic process benefits are associated with the reduction of unit operations. Furthermore, the commercial application of the prototype process as an alternative for the recovery of the protein of interest is raised.

A direct comparison of the new proposed strategy with the existing process (reported by *Herrera et al*[3]) involving an excessive number of process steps, highlights the superiority of the current approach. The process conditions of the subsequent steps of ATPS extraction, ultrafiltration and precipitation described in the proposed strategy were established previously.[7] It is clear that, for certain products, this bioengineering strategy opens the way to further bioprocess intensification. This novel process strategy greatly simplifies the traditional way in which biological products can be recovered, with significant scope for generic commercial application.

Conclusions

This study reports a process strategy for the potential production, release, and primary recovery with aqueous two-phase extraction of c-phycocyanin from *Spirulina maxima* in one single unit operation. It has been shown, that after broth conditioning, the release of c-phycocyanin can be achieved in the same fermentation flask. Furthermore, by adding the chemical forming phases of the ATPS, the cell disruption and the primary recovery of the protein can be performed simultaneously. It was demonstrated that a fermentation flask can be used as a cell disruption device by defining the appropriate operating conditions (e.g. glass beads diameter and loading, speed of agitation, $CaCl_2$ and biomass concentration). The operating conditions established for the cell disruption step together with those of the ATPS extraction resulted in one-stage process for the potential recovery of c-phycocyanin (with a purity of 1.7) from *Spirulina maxima*. The recovery process developed involved the direct integration of cell disruption in a fermentation flask that mimicked a high speed bead mill device and ATPS extraction. Overall, the results reported here demonstrated the potential application of a process integration strategy for the recovery of c-phycocyanina as a first step for the development of a commercial process.

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List of symbols

t – time, min
w – mass fraction, %
γ – mass concentration, g L^{-1}
φ – volume fraction, %
ω – circular frequency, min^{-1}
Y – yield, mg g^{-1}
X – number fraction, %

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