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(71) Applicant *(for all designated States except US)*: **ELI LILLY AND COMPANY** [US/US]; Lilly Corporate Center, Indianapolis, IN 46285 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **HARTMAN, Brian, Edward** [US/US]; 4444 North Washington Boulevard, Indianapolis, IN 46205 (US). **STROBEL, Robert, Joseph, Juni** [US/US]; 13807 Mill Stream Court, Carmel, IN 46032 (US). **SULLIVAN, Gary, Robert** [US/US]; 9022 Sargent Creek Drive, Indianapolis, IN 46256 (US).

(74) Agents: **PARKER, Raymond, S.** et al.; Eli Lilly and Company, Lilly Corporate Center, Drop Code 1104, Indianapolis, IN 46285 (US).

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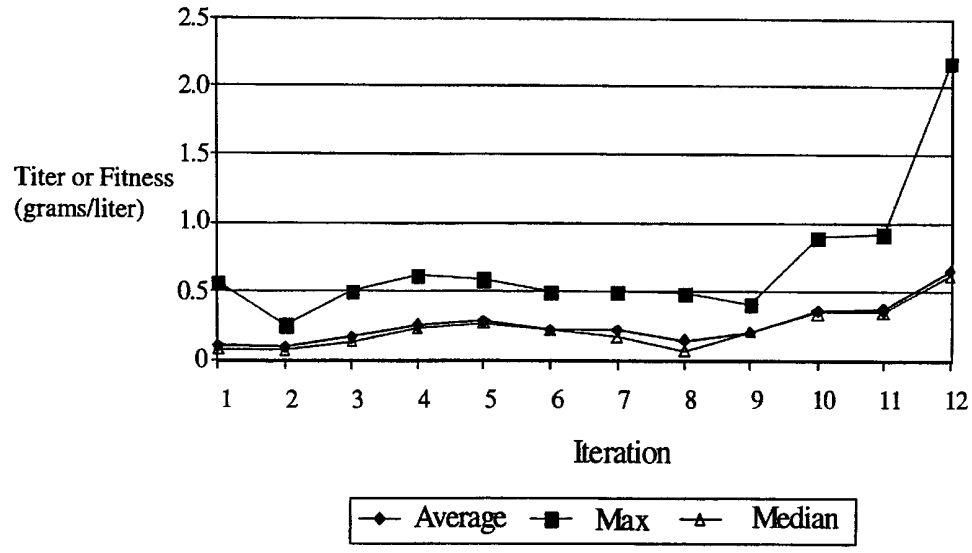
(54) Title: IMPROVED FERMENTATION PROCESS



(57) Abstract: The present invention provides a process for optimizing a fermentation process for the production of a glycopeptide nucleus by means of particle swarm optimization. In addition, an improved fermentation medium for the production of the glycopeptide nucleus was found which is significantly different and more potent than media identified by more conventional optimization methods.

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IMPROVED FERMENTATION PROCESS

FIELD OF THE INVENTION

The present invention relates to an improved fermentation process for the production of a glycopeptide nucleus from an *Amycolatopsis orientalis* or *Nocardia orientalis* medium based on particle swarm optimization.

BACKGROUND

Computational intelligence (CI) algorithms offer an alternative approach to *ad hoc* or statistically-designed optimization methods. The class of CI algorithms known as evolutionary algorithms(EA) seek to optimize an objective function by evolving a population of solutions in some manner. As their name suggests, biologically inspired operators such as crossover and mutation are used in combining good or "fit" solutions in order to improve the overall fitness of the population as an optimum is pursued. Stochastic elements of the search operators may lead to some portion of the population exploring very different, but feasible, regions of the search space. Solutions are not obtained, for example, by descending an objective function error gradient but rather by a pseudo-random search of the solution space. In general, no underlying assumptions about the objective function surface such as smoothness are necessary as they are with least-squares statistical design techniques.

The conceptual simplicity and ability to outperform classic methods in challenging real-world problems make CI algorithms attractive for study of fermentation media optimization. See e.g., Fogel D.B., "The Advantages of

Evolutionary Computation," In Proc. of BCE97: BioComputing and Emergent Computation, Lundh D, Olsson B, Narayanan A (eds.), Sinagapore:World Scientific, 1-11 (1997). However, it must be fairly noted that a "typical" application of these paradigms may involve very large population sizes (i.e. number of candidate solutions studied at each iteration) and may require many iterations to converge to an acceptable solution. Given that fermentation medium design can be expensive in terms of time, material, and labor costs, these are two potential drawbacks when considering the applicability of CI algorithms to medium design problems. Fortunately, these issues have not precluded research into the use of CI algorithms for this class of problem. In a similar application, Weuster-Botz and Wandrey used a GA to optimize fourteen medium ingredients for the formate dehydrogenase fermentation, requiring only four iterations. See, e.g., Weuster-Botz, D., et al., 1995. "Medium Optimization by Genetic Algorithm for Continuous Production of Formate Dehydrogenase," Process Biochemistry, **30**(6), 563-571 (1995). Similarly, Backhouse, *et al.* compared a GA with statistical techniques in the optimization of four parameters in a yarn-spinning process (five iterations), noting the importance of a good initial randomization of the population. See, e.g., Backhouse, P.G., et al., "A comparison of a genetic algorithm with an experimental design technique in the optimization of a production process," Journal of the Operational Research Society, **48**, 247-254 (1997).

To date, no one has attempted to optimize the fermentation process for production of glycopeptide nuclei using PSO. A variety of glycopeptide derivatives have been shown to have significant activity as an antibacterial

agent. See, e.g., U.S. Patent Nos. 4,643,987; 4,698,327; 5,840,684; 5,843,437; and 5,843,889. Given the complexity of components in a fermentation process, there is a need for identifying optimized parameters for the production of the glycopeptide nucleus for use in synthesizing glycopeptide antibacterial agents without having to conduct overwhelming experimentation.

SUMMARY

The present invention provides a process for optimizing a fermentation process for the production of a glycopeptide nucleus (e.g., A82846B) from *Amycolatopsis orientalis* or *Nocardia orientalis* (including mutants, variants or recombinants thereof) comprising the step of determining key component concentrations in the process by means of a particle swarm optimization.

In another embodiment of the present invention, an improved fermentation medium is provided wherein the improvement is characterized by optimization of key components of the medium by means of a particle swarm optimization. The composition of the improved fermentation medium as a result of applying the particle swarm optimization is also provided.

In yet another embodiment of the present invention, a fermentation medium for the production of a glycopeptide nucleus from *Amycolatopsis orientalis* which contains no animal source material (ASM), referred to herein as "ASM-free" fermentation medium. The ASM-free medium comprises cane molasses, hydrolyzed soybean flour and yeast. In a preferred embodiment, the medium also includes corn gluten.

Definitions

As used herein, the term "particle swarm optimization" or "PSO" refers to a computational intelligence algorithm as described in Eberhart, R.C., et al. , "A new optimizer using particle swarm theory," In Proc. Sixth Intl. Symposium on Micro Machine and Human Science, (Nagoya, Japan), Piscataway, IEEE Service Center, 39-43 (1995); Kennedy, J., "The particle swarm: Social adaptation of knowledge," In Proc. 1997 IEEE International Conference on Evolutionary Computation (Indianapolis, IN), Piscataway:IEEE Service Center, 303-308 (1997), and Eberhart, R., et al., "Evolutionary Computation Implementations," Computational Intelligence PC Tools, Boston, AP Professional, 212-226 (1996), and are hereby incorporated herein by reference, to the extent that they explain, further enable, provide a basis for or describe the subject matter to which is referred to in the specification.

"Key components" refer to chemical ingredients of the fermentation process that have significant effects on the yield of the desired product (e.g., glycopeptide nucleus).

"Titer" refers to the standard of strength of a volumetric test solution, i.e., the assay value of an unknown measure by volumetric means.

DESCRIPTION OF THE FIGURES

Figure 1 represents the PSO Average and Maximum Titer by Iteration.

DETAILED DESCRIPTION

A significantly improved medium for the production of a glycopeptide nucleus from *Amycolatopsis orientalis* was found using particle swarm optimization. Eleven medium

ingredients were optimized using a total of 240 shake-flask fermentations (12 iterations with a population size of 20; each "individual", or "agent" in the population represents a shake flask fermentation). The best medium improved glycopeptide titer > 2-fold over *ad hoc* experimentation. Whereas, a traditional statistical design approach using similar numbers of shake-flasks improved the titer 40% by optimizing five ingredients screened from the original 11. Surprisingly, the PSO approach yielded a different optimal medium than the medium derived through statistically designed experimentation.

PSO is a recent addition to the CI field. It is similar to well known EAs such as genetic algorithms but is defined in a social context as opposed to a biological context. The individuals in the population retain memory of known good solutions as they continue to search for better solutions, unlike EAs where knowledge is destroyed between generations. Interaction among parameters is thought to enhance the ability of the PSO algorithm to find good solutions. PSO seems to offer a powerful yet simple-to-implement paradigm with a maximum of two algorithm parameters that must be set prior to its use. This simplicity makes the PSO algorithm appealing as a starting point for forays into solving "real world" problems with CI algorithms. Finally, concerning the algorithmic issues above, PSO is reported to work well with relatively small population sizes and to converge quickly over the range of problems to which it has been applied to date (i.e. determining artificial neural network weights to solve the XOR problem. See, e.g., Eberhart, R.C., et al., "A new optimizer using particle swarm theory," In Proc. Sixth Intl. Symposium on Micro Machine and Human Science, (Nagoya,

Japan), Piscataway, IEEE Service Center, 39-43 (1995) and Eberhart R., et al., "Evolutionary Computation Implementations," Computational Intelligence PC Tools, Boston, AP Professional, 212-226 (1996).

The usual method of optimizing early-phase fermentation media is via the application of statistical design (SD) methods, such as screening and response surface experiments. These are inherently local optimization methods. PSO is a global optimization method and takes advantage of the intuitive fact that there are likely several 'optimal' fermentation mediums which may be located in the fermentation ingredient search space.

Applicants explored the applicability of PSO to fermentation medium optimization for production of glycopeptide nucleus in an attempt to identify advantages of using an alternative approach to statistical design (SD) methods. Classical *ad hoc*, or one-factor-at-a-time, experimentation was also conducted for comparison purposes. This work was done as an extension of efforts being made to replace an animal-source nutrient (pork skin residue) in a previously developed process for making the glycopeptide nucleus.

Applicants have discovered that the use of PSO provides several advantages, such as (i) lack of sensitivity to (initial) ingredient ranges, (ii) ability to optimize in higher dimensions, and (iii) global search vs. sequential experimentation.

The range of each ingredient to be included in the optimization study is typically set by the fermentation scientist prior to designing the experiments. The fermentation scientist balances knowledge of ingredient ranges which have been successful in the past with the

desire to explore broader ranges, keeping in mind the need to minimize the number of experiments to keep labor and material costs low and to meet ever more aggressive product development time-lines. One advantage of SD is its ability to alert the experimenter that an optimum does not lie in the design space currently being studied and to indicate where the design space must be moved to pursue an optimum. But the need to run additional experiments is required, possibly delaying the project and adding additional costs. An optimization paradigm more robust to initial ingredient ranges could offer advantages from the standpoint of not requiring additional screening experiments.

As mentioned above, applying SD to a problem with more than four or five independent variables typically requires a preliminary screening design in order to reduce the number of independent variables and keep the factorial load to a manageable level. Often, the results of the screening experiments are straightforward. For example, in a fermentation medium optimization experiment, ingredients that have a significant negative linear effect with respect to the objective function (titer) may be effectively removed from further consideration. But often the analysis of screening experiment requires the aid of an experienced statistician. For example, does an ingredient with a slightly negative main effect still warrant inclusion in the experiments because it has a slightly positive interaction with another ingredient? Consider also the case where several ingredients exhibit similar effects on the objective function at the screening experiment phase or where higher order interactions are suspected. Successful resolution to the question of which ingredients to remove and which ingredients to keep for further study can be crucial to

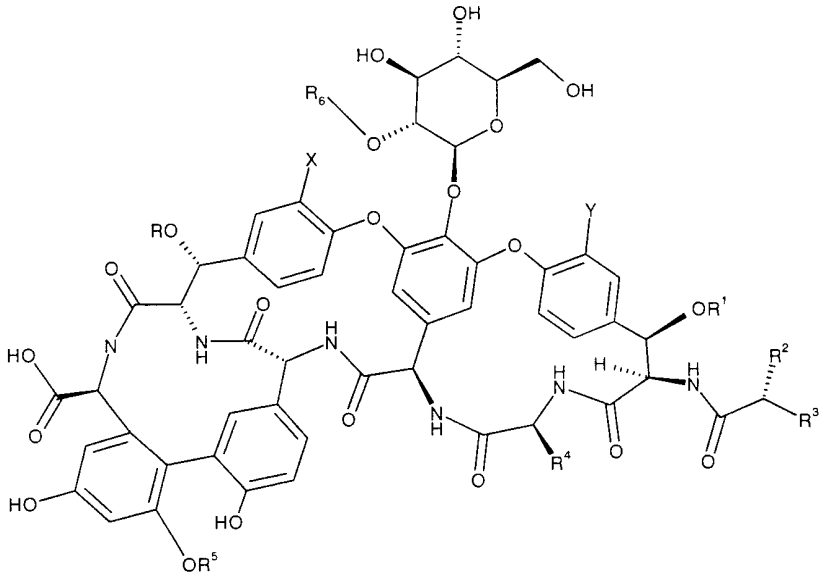
success of the project. Either bypassing the screening stage, and thus avoiding any decisions regarding whether or not to keep an ingredient in the study, or screening out only major negative effects and keeping the remaining ingredients in the plan could be advantageous.

SD techniques are sequential in nature - each stage of the experiment requires success in the prior stage(s). While conceptually simple, the proper application of SD methodologies can become technically demanding and require the assistance of a professional statistician to ensure success. Often some subjective decisions may have to be made when planning the next stage of experiments depending on the strength of the results from the just completed experimental stage. The experimenter must balance the need to "be bold" in planning further experiments with the constraint of operating in the current design space.

In a fermentation medium optimization problem with several ingredients it is not uncommon that more than one optimal solution exists - different mixtures of ingredients may provide similar titer results. Optimizing globally via a "population of solutions" may provide the researcher with not only an optimized fermentation medium, but with several candidate media to explore as the individuals move throughout the search space. Sequential optimization techniques like SD may also provide more than one candidate optimum, but these optima will tend to lie close together due to the local nature of the search process. The "richness" of solutions is another facet of the evolutionary algorithmic approach to be explored in this work. To summarize, the intent was to apply a novel CI algorithm to a "standard" problem typically solved with SD approaches. The ability of PSO to simplify the optimization process with

respect to ingredient range selection, preclusion of any screening stages, and the ability to globally optimize the objective function in high dimensions, thus possibly providing a "richer" set of solutions were of interest to Applicants.

The following examples illustrate the optimization of the fermentation process for the production of A82846B glycopeptide nucleus from using PSO. The A82846B glycopeptide nucleus has the following structure:



wherein R and R^6 are 4-*epi*-vancosaminyl, R^1 is hydrogen, R^2 is $NHCH_3$, R^3 is $CH_2CH(CH_3)_2$, R^4 is $CH_2(CO)NH_2$, R^5 is hydrogen, and X and Y are Cl. The A82846B glycopeptide nucleus may alternatively be produced from *Nocardia orientalis* strains (e.g., NRRL 18098, NRRL 18099, and NRRL 18100) which is described in U.S. Patent No. 5,312,738, incorporated herein by reference. Although the following Examples illustrate the use of PSO to optimize the fermentation media from a specific strain of *Amycolatopsis orientalis*, those skilled in the art will appreciate that other strains and/or mutants, variants or recombinants thereof may be optimized using this process as well. For

example, one may use mutant strains that are produced using Ultraviolet (UV) mutation procedures well known to those skilled in the art. One may select a specific mutant based on pigmentation which is also well know to those skilled in the art.

EXAMPLES

Preparations

Glycopeptide Vegetative Flask Medium (GV-003):

The GV-003 broth was prepared by combining the following components in the listed respective amounts.

Component	QA	Grams/Liter	% w/v
Glucose	QA020N	40	4
Phytone Peptone (BBL)	NA	15	1.5

No pH adjustment

Glycopeptide Bump Medium (GB-002):

The GB-002 broth was prepared by adding the following ingredients in the listed order. The pH was adjusted to 7.0 before addition of the calcium carbonate.

Component	QA/QD	Grams/Liter	%w/v
Glucose	QA020N	10	1
Corn Starch Powder	QA055L	5	0.5
Basic Yeast	QA220M	5	0.5
Hy-Soy™ (Quest)	QD463T	5	0.5
$CaCO_3$	QA209P	1	0.1

Fermentation Media:

The producing culture, an UV mutated strain from *Amycolatopsis orientalis*, was preserved and stored in liquid nitrogen. Cultures were thawed and inoculated into GV-003

broth to initiate a seed train for the PSO studies. After 48 hours, a small amount (0.1 mL) of GV-003 culture was transferred to second-stage seed broth, GB-002. After 48 hours growth in GB-002, 1.0 mL aliquots were transferred to flasks containing 50 mL of media designed through the PSO algorithm. All PSO media were buffered (pH 6.7) with 200 mM filter-sterilized 3-(N-morpholino)propanesulfonic acid (MOPS) added after flasks had been autoclaved (20 min.) and cooled. PSO cultures were grown at 34.5°C for 6 days in a shaker incubator set at 250 rpm with a relative humidity of 70%. Once a given set of shake-flask cultures was completed (one iteration), samples were taken from the flasks and sent to analytical laboratories for quantitative HPLC analysis of glycopeptide factors. A complete fermentation cycle, including preparation, inoculation, fermentation, assay, and setting a culture for the next round of experiments, required about 14 days.

PSO Methods

Basic algorithm:

Given a population of N individuals each individual i, $i \in \{1,\ldots,N\}$, has associated with it a position vector X_i on D dimensions. The quantity $X_i[d]$, $d \in \{1,\ldots,D\}$, represents the d^{th} parameter setting for individual i. The dimension D is problem dependent and depends upon the number of free parameters to be optimized in the system. At each iteration k an individual's position vector $X_i(k)$ is updated by adding a $\Delta X_i(k)$ vector, denoted V_i, to the current position. The vector V_i represents an individual's velocity in the D-dimensional problem hyperspace. Optimization is effected by modifying the V_i vectors of all the individuals in the population as shown below in equation 1.

Equation 1: Position update equation

$$X_i(k+1) = X_i(k) + V_i(k)$$

As the individuals, represented by particles, move throughout the problem space each retains knowledge of its best objective function value, denoted $pbest_i$ (for "personal best"), and the position in parameter hyperspace associated with that value of $pbest_i$, denoted $X_{pbest,i}$. The difference $X_{pbest,i} - X_i$ represents the geometric distance between an individual's current position and its best position found thus far. Adding a stochastic element in the form of a random positive number η results in an individual's update equation 2 given below.

Equation 2: Pbest velocity component update equation

$$V_i(k+1) = V_i(k) + \eta(X_{pbest,i} - X_i)$$

This update equation results in a stochastic tendency for each individual to return to its previous best position. This can be analogized with a human's tendency to remember and return to regions in the psychological space which have seemed beneficial or promising in the past.

To incorporate an interaction element the concept of a neighborhood is employed, defined as the nearest i - s and i + s individuals, for a neighborhood of size s, including the i^{th} individual. In the context of this neighborhood construct, each individual retains knowledge of the overall best value of the objective function found thus far for the entire neighborhood, denoted gbest (for "global best"). The position in the problem space associated with the global best value gbest is denoted X_{gbest}. The vector $X_{gbest} - X_i$ represents the distance from individual i's current position and the position associated with the overall best objective

function value found thus far in the individual's neighborhood. The interaction component of the algorithm then consists of an update to V_i for each individual as shown below in Equation 3.

Equation 3: Gbest velocity component update equation

$$V_i(k+1) = V_i(k) + \eta(X_{gbest,i} - X_i)$$

To prevent computer overflow as well as to define the granularity of the search a parameter defined as V_{max}, representing maximum velocity, is defined and incorporated into the algorithm. At each iteration the newly calculated value of V_i is compared to V_{max} and the minimum value is chosen for $V_i(k+1)$, preserving the sign of the current direction. Combining (2) and (3) yields the complete velocity update equation 4 below (note that the velocity may be positive or negative).

Equation 4: Velocity update equations

$$V_i(k+1) = MIN\{V_i(k) + \eta_1(X_{pbest,i} - X_i) + \eta_2(X_{gbest,i} - X_i), V_{max}\}$$

Metaphorically, individuals (represented by particles) are flown through D-dimensional hyperspace in search of good solutions. Individuals are guided in their search by knowledge of where the best solutions have been found thus far. The preservation of information between iterations sets the PSO algorithm apart from many evolutionary algorithms where information is typically destroyed between generations. (*Elitism*, or the introduction of one of the most fit individuals in generation *k* into generation *k+1* is an information preservation technique sometimes used in evolutionary algorithms, though not usually on the scale of PSO). The construct of the update equations ensures

individuals will 'fly by' known good regions and allocate some search time to unknown regions.

The PSO algorithm, was coded as a MATLAB (The MathWorks, Cambridge, MA) m-file. The algorithm was written to accept tab-delimited ASCII files containing information about the current iteration, such as coded ingredient levels, current ingredient delta values (e.g. agent velocities), values and location of each individual's best fitness (pbest values), the titer results of the current fermentation (e.g. current fitness values), etc. New files are output containing the next set of experiments. In this manner the algorithm was run one iteration at a time, dependent upon the arrival of updated fitness information from the current set of experiments.

Microsoft Excel (Microsoft Corp., Redmond, WA) and JMP (The SAS Institute, Cary, NC) software were used to manipulate the various data files generated, for example de-scaling the coded ingredient levels returned by the PSO algorithm into the actual ingredient concentrations in grams/liter. The various algorithmic details are described next.

Setting up the search space:

The number of ingredients as well as the allowable range of each ingredient defines the ingredient space in which the PSO population will operate. The determination and application-related issues of each are discussed below.

Number of ingredients: The fermentation medium initially provided contained six ingredients, including a complex animal-source nutrient. Based on the composition of the current medium, the desire to remove the animal-source

raw material, and overall production cost targets, the development scientist selected 11 ingredients for inclusion in the study.

Ingredient ranges: The development scientist, using his judgement and prior experience, sets an initial range for each ingredient. These initial ranges were then linearly coded into [-10.0,10.0] for use in the PSO algorithm. For PSO, the determination of ingredient ranges is primarily to allow for initialization of the population (see below) and to start the PSO population in a space where there is a reasonable chance of success.

Ingredient levels below 0.0 (uncoded) are infeasible. Thus, during the position update step of the algorithm, if any ingredient fell below -10.0 (coded), it was then clamped to -10.0 (coded). A question arises as to what to do about the velocity value for an ingredient when it is clamped to its minimum value. Consider an ingredient that is heading towards its minimum value (implying negative velocity) and reaches the lower bound. If the individual attempted to move to a different portion of the ingredient space including non-zero values of the ingredient, some time may be 'wasted' in that at least one iteration would likely be required to change the value of the velocity from negative to positive, after which the ingredient level could 'move away' from 0.0. For this reason, any time an ingredient was clamped to its lower bound, the associated velocity was set to 0.0.

Conversely, the upper end of the ingredient ranges was set to 100.0 (coded), essentially providing for an unbounded search of the ingredient space. The constraints above

result in the modifications to the position and velocity update equations shown below.

Equation 5: Modified position update equation

$$X_i(k+1) = MAX\{X_i(k) + V_i(k), -10.0\}$$

Equation 6: Modified velocity update equation

$$V_i(k+1) = MIN\{V_i(k) + \eta_1(X_{pbest,i} - X_i) + \eta_2(X_{gbest,i} - X_i), V_{\max}\}$$

If $X_i(k+1) \leq -10.0$ then $V_i(k+1) = 0.0$ and $X_i(k+1) = -10.0$

Setting Vmax:

Vmax was set to +/- 20% of the coded initial ingredient ranges, or +/-2.0. This is commensurate with the typical 'rules of thumb' for using PSO and other computational intelligence tools with a similar granularity construct.

Population size:

A population size of 20 was selected for this work. It was felt this population size would allow for a complete trial (consisting of 20 shake-flasks) to be prepared in 4-8 hours depending on the number of resources dedicated to the effort. Minimizing the amount of effort required for preparing the PSO shake-flask media was considered important in light of the high workload in the area.

Number of iterations:

It was hoped the project would run through 15 iterations of experiments. Although 15 iterations is certainly a 'few' iterations in the context of applying computational intelligence algorithms, it still represented a fairly large amount of development time (15x20 = 300 planned experiments). One way to explore the reduction of

overall development time is to investigate the application of PSO and SD methods in a hybrid sense. To address this second project goal, the selection of a 'novel' location in ingredient space would be done after the sixth iteration. This would provide the PSO algorithm five iterations (in addition to initialization) before the selection of a medium was made.

Objective function:

Much discussion centered on how many project goals to encode in the objective function. In addition to product titer, broth viscosity was another response factor considered for inclusion into the objective function. Another metric considered was the percentage of the desired chemical entity secreted by the *A. orientalis* production culture, as at least two other chemical analogs were usually produced in addition to the analog of interest. To meet downstream purification targets, the desired chemical entity had to represent a certain percentage (61%) of the overall amount of the three analogs produced.

Several candidate objective functions were considered to produce a figure of merit that would seek to reward higher titer, lower viscosity, and meeting or exceeding the percentage goal for the desired glycopeptide factor. The possibility of simply having the scientist rank-order each set of experiments was considered as well. In the end, concerns about trying to achieve too many goals at once during an initial foray into this type of project led the team to decide to keep the objective function straightforward, including only the potency metric. It should be said that often media that provide for good titer values usually have the other desirable characteristics as

well. While serendipity may come into play with respect to the achievement of these ancillary goals, they are also somewhat interrelated. For example, fermentation media which become very viscous over the course of the fermentation are less likely to promote good culture growth and chemical synthesis as the high viscosity provides a less than optimal environment due to impediments on mixing and oxygen mass transfer. Therefore, fermentation media that provide for high culture growth and product synthesis generally must usually exhibit favorable viscosity characteristics in order to achieve the high growth and synthesis in the first place.

Samples were taken twice during the course of a PSO iteration to measure glycopeptide titer (potency), and it was decided to scale the titer values. The resulting objective function used to score the fitness of the PSO population after each trial was then:

Equation 7: Titer (Potency) Objective Function

$$\frac{Max(Potency-sample-1, Potency-sample-2)}{1000}$$

Neighborhood size:

Keeping with the goals of the experiment, it was decided to use an initial neighborhood size of 3 individuals. After five iterations, the neighborhood size would be increased to 5 individuals. The neighborhood size would then be increased to its final value of 7 (or possibly more) for the last five iterations. The basic intent was to use a more 'localized' version of the algorithm for the first part of the project, with the goal being to promote search of several regions of the ingredient space at once.

As the algorithm progressed and good solutions were found in the various regions of ingredient space - the 'neighborhoods' - the algorithm would become more global in nature, with the best solution(s) having a greater impact on a larger portion of the PSO population.

Initialization of algorithm:

In CI applications it is common practice to initialize the population at random locations in the problem space, and the authors have used this approach in the past. For this work a different approach was used, following from the experience that CI algorithms typically require several iterations to become organized, after which real progress is made. The initialization of the population was thus effected in a manner similar to the generation of a screening design, with the intent to make the rows and columns of the design matrix as orthogonal and independent as possible. The design matrix used to initialize the PSO experimentation is provided in Table 1 below.

Table 1

Initial PSO design matrix (ingredients in grams per liter)

	Glucose	$MgSO_4$	$(NH_4)_2SO_4$	KH_2PO_4	KCl	Cane Molasses	Corn Gluten Meal	Hy-Soy™	Basic Yeast	Cotton Seed Flour	Corn Steep Powder
Flask 1	27.5	5.0	7.5	0.4	3.3	15.0	22.5	1.3	2.5	15.0	2.5
Flask 2	10.0	20.0	10.0	0.0	1.0	60.0	0.0	5.0	10.0	0.0	0.0
Flask 3	10.0	20.0	10.0	0.5	10.0	0.0	0.0	5.0	0.0	20.0	10.0
Flask 4	10.0	0.0	10.0	0.0	10.0	60.0	30.0	0.0	10.0	20.0	0.0
Flask 5	62.5	15.0	2.5	0.1	3.3	15.0	22.5	1.3	7.5	15.0	2.5
Flask 6	27.5	5.0	2.5	0.1	7.8	15.0	7.5	2.5	5.0	15.0	7.5
Flask 7	27.5	15.0	2.5	0.1	3.3	45.0	7.5	3.8	7.5	15.0	2.5
Flask 8	62.5	15.0	7.5	0.1	3.3	15.0	22.5	3.8	2.5	5.0	7.5
Flask 9	10.0	20.0	0.0	0.0	10.0	0.0	30.0	3.8	2.5	0.0	10.0
Flask 10	62.5	15.0	7.5	0.4	7.8	45.0	7.5	0.0	10.0	5.0	2.5
Flask 11	27.5	5.0	7.5	0.4	3.3	15.0	7.5	1.3	7.5	5.0	7.5
Flask 12	80.0	0.0	0.0	0.5	1.0	60.0	30.0	1.3	7.5	20.0	10.0
Flask 13	80.0	0.0	10.0	0.0	10.0	0.0	30.0	2.5	5.0	0.0	0.0

	Glucose	$MgSO_4$	$(NH_4)_2SO_4$	KH_2PO_4	KCl	Cane Molasses	Corn Gluten Meal	Hy-Soy™	Basic Yeast	Cotton Seed Flour	Corn Steep Powder
Flask 14	80.0	0.0	10.0	0.0	1.0	60.0	0.0	5.0	0.0	20.0	10.0
Flask 15	27.5	5.0	2.5	0.4	7.8	45.0	22.5	0.0	0.0	5.0	2.5
Flask 16	10.0	20.0	0.0	0.5	1.0	60.0	30.0	3.8	2.5	0.0	10.0
Flask 17	80.0	20.0	0.0	0.5	10.0	0.0	0.0	0.0	0.0	20.0	0.0
Flask 18	80.0	0.0	0.0	0.5	1.0	0.0	0.0	0.0	0.0	0.0	0.0
Flask 19	45.0	10.0	5.0	0.3	5.5	30.0	15.0	5.0	10.0	10.0	5.0
Flask 20	62.5	5.0	2.5	0.1	7.8	45.0	7.5	2.5	5.0	5.0	7.5

Following the first experiment (above), the design matrix was reorganized in an attempt to take advantage of the neighborhood construct to be employed. A 20x11 matrix was constructed consisting of inter-column Euclidean distances. Using the first individual as a starting point, the individual with the least calculated distance from the first was re-indexed to be the second individual in the population. Next, the individual the least distance from the second individual was moved and re-indexed as the third individual, and so on. In this manner each neighborhood consisted initially of individuals with the least distance from each other in ingredient space. Table 2 reflects the updated distance-based design matrix.

Table 2

PSO position initialization (ingredients in grams per liter)

	Glucose	$MgSO_4$	$(NH_4)_2SO_4$	KH_2PO_4	KCl	Cane Molasses	Corn Gluten Meal	Hy-Soy™	Basic Yeast	Cotton Seed Flour	Corn Steep Powder
Flask 1	27.5	5.0	7.5	0.4	3.3	15.0	22.5	1.3	2.5	15.0	2.5
Flask 2	27.5	5.0	2.5	0.1	7.8	15.0	7.5	2.5	5.0	15.0	7.5
Flask 3	27.5	5.0	7.5	0.4	3.3	15.0	7.5	1.3	7.5	5.0	7.5
Flask 4	45.0	10.0	5.0	0.3	5.5	30.0	15.0	5.0	10.0	10.0	5.0
Flask 5	27.5	15.0	2.5	0.1	3.3	45.0	7.5	3.8	7.5	15.0	2.5
Flask 6	27.5	5.0	2.5	0.4	7.8	45.0	22.5	0.0	0.0	5.0	2.5
Flask 7	10.0	20.0	0.0	0.5	1.0	60.0	30.0	3.8	2.5	0.0	10.0
Flask 8	10.0	0.0	10.0	0.0	10.0	60.0	30.0	0.0	10.0	20.0	0.0
Flask 9	10.0	20.0	10.0	0.0	1.0	60.0	0.0	5.0	10.0	0.0	0.0
Flask 10	62.5	5.0	2.5	0.1	7.8	45.0	7.5	2.5	5.0	5.0	7.5
Flask 11	62.5	15.0	7.5	0.4	7.8	45.0	7.5	0.0	10.0	5.0	2.5

	Glucose	$MgSO_4$	$(NH_4)_2SO_4$	KH_2PO_4	KCl	Cane Molasses	Corn Gluten Meal	Hy-Soy™	Basic Yeast	Cotton Seed Flour	Corn Steep Powder
Flask 12	62.5	15.0	7.5	0.1	3.3	15.0	22.5	3.8	2.5	5.0	7.5
Flask 13	62.5	15.0	2.5	0.1	3.3	15.0	22.5	1.3	7.5	15.0	2.5
Flask 14	80.0	0.0	10.0	0.0	10.0	0.0	30.0	2.5	5.0	0.0	0.0
Flask 15	80.0	0.0	0.0	0.5	1.0	0.0	0.0	0.0	0.0	0.0	0.0
Flask 16	80.0	20.0	0.0	0.5	10.0	0.0	0.0	0.0	0.0	20.0	0.0
Flask 17	80.0	0.0	10.0	0.0	1.0	60.0	0.0	5.0	0.0	20.0	10.0
Flask 18	80.0	0.0	0.0	0.5	1.0	60.0	30.0	1.3	7.5	20.0	10.0
Flask 19	10.0	20.0	0.0	0.0	10.0	0.0	30.0	3.8	2.5	0.0	10.0
Flask 20	10.0	20.0	10.0	0.5	10.0	0.0	0.0	5.0	0.0	20.0	10.0

The velocity, or change in position, for each ingredient in each flask must also be initialized, and again it is common practice to initialize the PSO velocities in some random manner. For this work it was considered to initialize all the velocities to 0.0, again in the context of trying to give the PSO population the most efficient starting point and (lack of) initial trajectory. It was thought that perhaps a random initialization of the velocities might contribute to the PSO population requiring a several iterations simply to organize, such as when the combined velocity vector led an agent to a worse-performing region of ingredient space, from which it would seek to return. However, Applicants eventually decided to initialize the velocities to $N(0,V_{max})$ random values. Table 3 contains the initial velocity settings for all agents.

Table 3

PSO velocity initialization (coded values)

	Glucose	$MgSO_4$	$(NH_4)_2SO_4$	KH_2PO_4	KCl	Cane Molasses	Corn Gluten Meal	Hy-Soy™	Basic Yeast	Cotton Seed Flour	Corn Steep Powder
Flask 1	0.23	1.75	-1.61	0.28	1.48	1.85	0.00	-1.72	0.10	-1.62	-1.33
Flask 2	-0.49	-0.16	-1.46	-0.37	-0.26	-0.64	-1.11	-0.46	1.62	1.77	-1.09
Flask 3	-1.30	1.87	-0.80	-1.31	1.73	-0.11	1.76	1.83	1.27	1.50	1.58
Flask 4	-1.72	-1.21	0.31	-0.77	-1.96	1.64	-1.97	-0.92	1.39	1.20	0.67
Flask 5	-0.72	-0.12	1.72	1.21	0.63	-0.06	0.90	-0.59	1.81	1.94	-1.35
Flask 6	1.29	-1.04	0.62	1.00	-0.92	0.76	1.72	0.41	-0.34	1.47	-1.44

	Glucose	$MgSO_4$	$(NH_4)_2SO_4$	KH_2PO_4	KCl	Cane Molasses	Corn Gluten Meal	Hy-Soy™	Basic Yeast	Cotton Seed Flour	Corn Steep Powder
Flask 7	-1.65	1.65	-1.28	0.43	-0.15	0.57	1.40	1.08	1.49	0.22	0.77
Flask 8	-1.67	0.76	1.05	1.84	1.17	-0.67	0.67	1.26	-1.83	0.76	-0.29
Flask 9	0.59	1.37	-0.49	1.78	1.78	0.01	-0.21	0.29	-1.47	0.75	-1.00
Flask 10	1.46	0.26	0.34	0.39	-1.80	1.88	-0.29	-1.03	-0.97	-0.01	0.78
Flask 11	-1.64	-1.11	0.07	-0.08	0.49	-0.08	0.08	-0.73	-0.04	0.12	-1.99
Flask 12	-0.68	0.87	-1.33	-0.75	1.23	-1.36	0.00	-0.96	-1.57	-1.59	1.88
Flask 13	-1.02	-1.53	-0.11	-1.08	0.99	1.54	1.97	0.40	-1.33	0.55	1.18
Flask 14	-1.31	-0.23	-1.10	1.66	0.00	-0.67	0.16	0.95	0.78	0.24	1.89
Flask 15	-1.00	-0.08	-1.17	0.16	-0.07	-0.09	1.29	0.85	0.29	1.75	-0.67
Flask 16	-1.85	0.63	-0.81	-0.42	-0.14	-0.89	1.44	-0.85	-0.77	-1.78	0.30
Flask 17	0.02	0.75	-1.33	-1.72	0.30	-1.72	1.40	0.16	0.44	0.76	1.77
Flask 18	0.11	0.22	0.71	0.78	-0.52	-0.90	-0.55	0.48	1.82	-1.37	0.67
Flask 19	-1.95	-1.81	-0.66	-1.85	-1.71	-1.59	0.08	1.35	-1.34	-0.83	1.05
Flask 20	1.68	1.72	1.72	-1.12	-0.42	-0.08	-1.70	0.19	-0.70	-0.77	-1.76

Velocity damping:

Finally, one additional modification was made to the velocity update equation. The velocity is multiplied by a weight, or damping factor, which declines linearly over several iterations. For this work, the weight was initialized to 0.9 and the intent was to reduce the weight to 0.4 over 15 iterations. Similar to the momentum term in standard back-propagation of error optimization algorithms, the weight term works to 'smooth' movement in ingredient-space which could be helpful in finding any optima once promising regions are located. Considering this weight term, the final velocity update equation results, as given below.

Equation 8: Final velocity update equation

$$V_i(k+1) = MIN\{w[V_i(k) + \eta_1(X_{pbest,i} - X_i) + \eta_2(X_{gbest,i} - X_i)], V_{\max}\}$$

$$\text{If } X_i(k+1) \le -10.0 \text{ then } V_i(k+1) = 0.0 \text{ and } X_i(k+1) = -10.0$$

The results of applying PSO to glycopeptide optimization fermentation medium are described below. The results of maximizing potency are presented first and

compared to the results achieved in the parallel SD optimization work.

It should be noted that in the middle of the experiment it was determined that a key ingredient, $(NH_4)_2SO_4$, had become contaminated and had likely adversely affected the results of several of the PSO iterations. This problem remained unnoticed for several iterations of the PSO algorithm. However, the excellent results obtained using PSO suggest the algorithm was able to easily recover once the ingredient problem was corrected.

The total number of PSO iterations were reduced to 12 rather than the planned 15. The progression of fitness values by iteration is illustrated in Table 4 below and Figure 1.

Table 4

PSO medium optimization results for each iteration

(g of nucleus/liter)

	1	2	3	4	5	6	7	8	9	10	11	12
Flask 1	0.06	0.07	0.12	0.14	0.28	0.29	0.12	0.19	0.09	0.13	0.37	0.30
Flask 2	0.02	0.23	0.10	0.12	0.27	0.22	0.17	0.41	0.20	0.24	0.23	0.34
Flask 3	0.00	0.15	0.26	0.11	0.21	0.07	0.18	0.04	0.11	0.13	0.20	0.20
Flask 4	0.15	0.10	0.11	0.21	0.21	0.05	0.23	0.00	0.32	0.30	0.47	0.59
Flask 5	0.11	0.03	0.07	0.12	0.33	0.31	0.29	0.05	0.36	0.39	0.49	0.52
Flask 6	0.00	0.09	0.49	0.24	0.32	0.25	0.49	0.00	0.32	0.24	0.92	0.77
Flask 7	0.00	0.00	0.19	0.19	0.27	0.01	0.40	0.23	0.34	0.64	0.69	0.94
Flask 8	0.00	0.01	0.00	0.10	0.00	0.06	0.01	0.00	0.34	0.46	0.55	0.71
Flask 9	0.16	0.18	0.21	0.61	0.59	0.30	0.41	0.27	0.20	0.50	0.50	0.74
Flask 10	0.13	0.08	0.01	0.28	0.25	0.50	0.26	0.35	0.41	0.89	0.51	0.74
Flask 11	0.56	0.14	0.01	0.44	0.45	0.21	0.22	0.49	0.21	0.32	0.41	0.67
Flask 12	0.21	0.04	0.45	0.29	0.44	0.07	0.16	0.03	0.03	0.26	0.22	0.57
Flask 13	0.02	0.04	0.04	0.44	0.10	0.10	0.15	0.04	0.02	0.12	0.05	0.22
Flask 14	0.14	0.07	0.40	0.16	0.05	0.05	0.04	0.07	0.21	0.24	0.33	2.18
Flask 15	0.04	0.07	0.13	0.42	0.40	0.45	0.12	0.04	0.41	0.49	0.11	0.79
Flask 16	0.09	0.10	0.18	0.30	0.35	0.22	0.17	0.15	0.11	0.42	0.20	0.71
Flask 17	0.06	0.01	0.00	0.00	0.20	0.39	0.16	0.03	0.04	0.36	0.27	0.64
Flask 18	0.16	0.00	0.00	0.00	0.17	0.12	0.08	0.04	0.05	0.13	0.11	0.54
Flask 19	0.02	0.25	0.21	0.31	0.43	0.44	0.44	0.35	0.19	0.52	0.44	0.51
Flask 20	0.14	0.07	0.24	0.50	0.23	0.28	0.15	0.15	0.06	0.42	0.29	0.36
Average	0.10	0.09	0.16	0.25	0.28	0.22	0.21	0.14	0.20	0.36	0.37	0.65

Median	0.07	0.07	0.12	0.23	0.27	0.22	0.17	0.06	0.20	0.34	0.35	0.61
Maximum	0.56	0.25	0.49	0.61	0.59	0.50	0.49	0.49	0.41	0.89	0.92	2.18

The period of ingredient contamination from iteration 5 through iteration 9 can be readily seen as the average population fitness decreases slightly during this period. Once the situation was corrected, the average and maximum fitness values increased dramatically. Average fitness increased from 0.1 g/L to 0.65 g/L, or 550%. Similarly, median fitness increased from 0.07 g/L to 0.61 g/L, or 771%. Discounting the highest fitness result obtained in the 12th (final) iteration, the mean and median fitness values increased 270% and 250%, respectively. The medium achieving the best fitness (2.18 g/L) in the final iteration was comprised of 10 of the 11 possible ingredients, with 3 of the ingredients present in very minute quantities. In all, 12 x 20 = 240 individual flask trials were performed, requiring approximately 7 months to complete. By these measures, the results achieved using PSO were excellent.

In comparison, the SD project work yielded a medium with a fitness of 1.05 g/L, requiring 7 ingredients. A response surface design suggested that a simpler medium, consisting of only 3 of the 7 ingredients, would achieve a predicted potency of 1.1 g/L. In all, approximately 100 SD shake-flask trials were performed, requiring approximately 4 months to complete.

Beyond the level of fitness achieved, the difference in composition of the optimal fermentation media obtained is of interest and useful for comparison. Table 5 lists the composition of the best PSO medium along with the best SD-derived fermentation medium and the best medium obtained by the *ad hoc* approach. The titers shown are averages for

several flask trials (replicates). The fact that the PSO and SD-derived media are very different is readily seen, although some similarities are evident as well. It is interesting to note the best PSO result was obtained in the ingredient space defined by the initial PSO ingredient ranges. In contrast, the design space moved under the SD approach, with Basic Yeast at 12.5 g/L in the best SD results. Examination of the PSO population locations over all the experiments (not shown) indicates several ingredients were tested outside the initial ranges, but the overall tendency was to search within the pre-defined ingredient space as that was where the best solutions were located by the PSO population.

Table 5

Comparison of optimized media[1]

	PSO Medium	SD Medium	*Ad Hoc* Medium	*ASM* Medium
Ingredient (g/liter)				
Meat Peptone	0.0	0.0	0.0	22.5
Glucose	80.0	45.0	45.0	45.0
$MgSO_4$	0.0	1.2	1.2	1.2
$(NH_4)_2SO_4$	7.3	4.13	4.13	4.13
KH_2PO_4	0.15	0.16	0.16	0.16
KCl	8.6	10.0	5.5	5.5
Cane Molasses	2.6	10.0	0.0	0.0
Corn Gluten	23.1	0.0	20	0.0
Hy-Soy™	2.5	2.5	0.0	0.0
Basic Yeast	4.9	12.5	0.0	0.0
Titer (g/L)	**1.8**	**1.05**	**0.74**	**1.0**
Std Deviation	**0.09**	**0.05**	**0.06**	**0.05**
No. of Replicates	**4**	**4**	**4**	**4**

[1]All media contained $CaCO_3$ at 5.2 grams/liter

In examining the above results, it is clear the PSO algorithm was able to optimize the fermentation medium for the modified glycopeptide process. In addition, the PSO algorithm was shown to be robust in the face of some challenging issues. For example, the incidence of an

ingredient becoming contaminated had no more a deleterious effect than the likely addition of iterations required to reach high glycopeptide titer. Although it is only conjecture, considering that iterations at risk consisted of 80-100 experiments, it is possible similar results might have been realized in 140-160 PSO experiments, or 7 to 8 trials, which is very similar to the number required for SD optimization. Furthermore, the ability to simply 'keep going', rather than having to 'go back' and repeat one or more experimental stages - as would likely be required in SD - is certainly of value.

This result represents a very exciting discovery for the area, not only with respect to the ability of a tool like PSO to provide an optimized fermentation medium, but also in terms of the value delivered to the project by providing a fermentation medium that supports high product titer. In addition, the optimized medium identified enhances the prospects for commercialization of a glycopeptide antibacterial agent by providing cost savings as a result of increased productivity of the medium.

In addition, a fermentation medium was identified which was free from animal source materials (ASM) without sacrificing titer. In fact, the PSO medium using a combination of cane molasses (or blackstrap molasses), corn gluten, Hy-Soy™ (hydrolyzed soybean flour available from Quest International, Norwich, NY) and yeast (e.g. Red Star basic yeast, available from Red Star, Milwaukee, WI) as a nutrient source provided a titer which was 180% higher than a comparative medium using a meat peptone as a nutrient source. The SD medium using cane molasses, Hy-Soy™ and basic yeast as a nutrient source provided a titer which was

nearly equivalent to the comparative medium using meat peptone (105%). Therefore, both the PSO and SD ASM-free media provide viable alternatives for media containing animal-source materials.

Other useful alternatives for cane molasses include any sucrose related materials. Useful alternatives for Hy-Soy™ include other hydrolyzed soybean flours which may be hydrolyzed enzymatically, or by means of an acid or base hydrolysis. Suitable yeasts include any spray dried whole yeast materials or yeast extracts. Suitable corn glutens include whole or hydrolyzed glutens and equivalents thereof.

CLAIMS

We Claim:

1. A process for optimizing a fermentation process for the production of a glycopeptide nucleus from *Amycolatopsis orientalis* or *Nocardia orientalis* comprising the step of determining key component concentrations in said process by means of a particle swarm optimization.

2. The process of Claim 1 wherein said glycopeptide nucleus is A82846B.

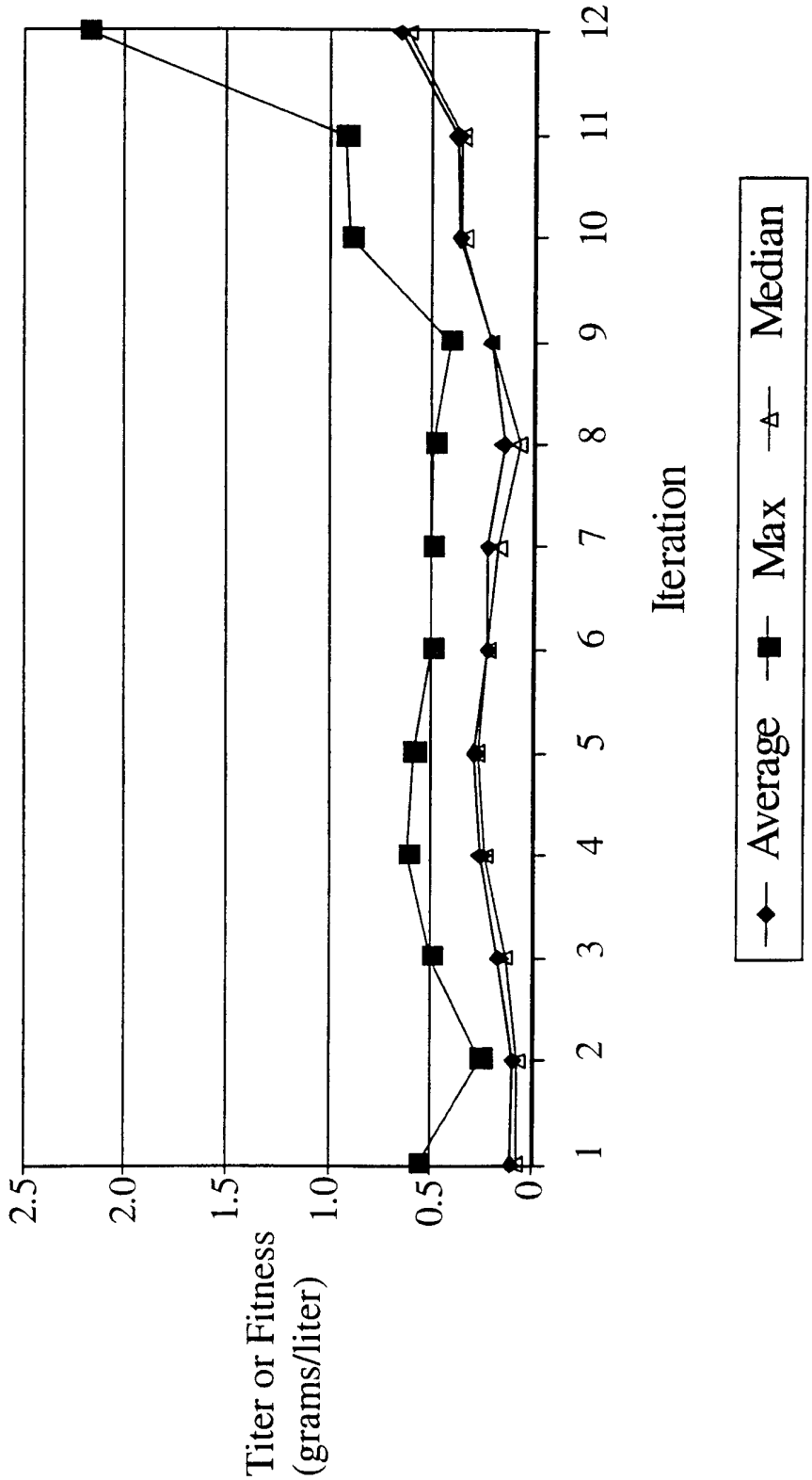
3. An improved fermentation medium for the production of a glycopeptide nucleus from *Amycolatopsis orientalis* or *Nocardia orientalis* wherein said improvement is characterized by optimization of key components of said medium by means of a particle swarm optimization.

4. The medium of Claim 3 wherein said glycopeptide nucleus is A82846B.

5. An ASM-free fermentation medium for the production of a glycopeptide nucleus from *Amycolatopsis orientalis* comprising cane molasses, hydrolyzed soybean flour and yeast.

6. The fermentation medium of Claim 5 further comprising corn gluten.

FIG. 1


Titer or Fitness
(grams/liter)
2.5
2.0
1.5
1.0
0.5
0
1
2
3
4
5
6
7
8
9
10
11
12
Iteration
Average
Max
Median