MECHANISM OF CITRIC ACID ACCUMULATION BY *ASPERGILLUS NIGER* IN SOLID STATE FERMENTATION

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Abstract

The main purpose of this work was to study the mechanism of citric acid accumulation in solid state fermentation of *Aspergillus niger*. Two strains, Yang No.2 and MH 15-15, represented the high-accumulating organisms from which the low-accumulating mutants, SL-1 and SL-2, were generated by ultraviolet treatment. Comparative solid state fermentations, with a starch-containing material as the substrate, were conducted in petri-dishes, a technique which conferred a major advantage in allowing recovery of metabolically active mycelia for biochemical assays. Apart from the decreased citric acid accumulation, the selected mutants displayed lower starch consumption and enhanced production of oxalic acid, while their growth were generally equal to that of their respective parents. Evidence on elevated levels of free glucose in the cultures of the mutants, despite there being no alteration of α-amylase and glucoamylase from their parents, has prompted a hypothesis that the mutants were defective in the rates of glucose uptake.

The biochemical work started with the primary steps of carbon assimilation, *viz* measurement of glucose uptake and activity assay of hexokinase. The results confirmed the reduced glucose uptake rates by the mutants and a hypothesis that this is caused by some defects in certain components of the glucose transport mechanism, but not at membrane ATPase, has been proposed. In addition, hexokinase showed higher *in vitro* activities in the parents and, presumably, their glycolytic fluxes were greater than those of their mutants.

Investigation of activities of some selected TCA cycle enzymes and other metabolic steps *in vitro* strongly indicated the decreased activity of 2-oxoglutarate dehydrogenase and, possibly, NAD-and NADP-specific isocitrate dehydrogenases in the parents. Although most other enzymes decreased their activities during the later phase of cultivation, there was no definite difference between each parent and its mutant. However, oxaloacetate hydrolase, for oxalate formation, was at higher activity in the mutants than in the parents.

Measurements of intracellular concentrations of products of certain enzymes and adenine nucleotides were conducted in order to assess the *in vivo* catalytic function of the enzymes of interest. It was concluded that internal accumulation of citrate or oxalate is an immediate cause of its excretion. Supplemented by evidence from the ratio of ATP/AMP in the cells, a complete hypothesis describing citrate accumulation in *A.niger* Yang No.2 and MH 15-15 is proposed. Hence, the rate by which glucose is taken up into the cells is the primary trigger determining the capacity of glycolytic metabolism and it is proposed that the primary cause of citric acid accumulation in the high-accumulating strains is the deregulation of glucose uptake. When the glucose supply exceeds the requirement of the cells, i.e. when growth is slow, the TCA cycle is balanced by allosteric deactivation of isocitrate dehydrogenases by ATP which is excessively generated *via* the active glycolysis. The observed low level of activity of 2-oxoglutarate dehydrogenase is, therefore, a result of this metabolic block, rather than a cause. Because of the equilibria of the reactions, citrate is accumulated and then excreted out of the cells. In contrast, when the glucose supply is below such level, this enzyme regulation does not occur and oxalate, instead of citrate, acts as the drain of excess carbon going around the fully operative TCA cycle.

In conclusion, the current hypothesis for citric acid accumulation is basically similar to that proposed for submerged fermentation conditions. However, the rationale for carbon sink *via* oxalate is novel. Finally, it has been shown that two *A.niger* strains of different origins displayed a similar mechanism, although the fine control may be different.

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Table of Contents

List of Figures

List of Tables

Abbreviations

Abbreviation of Units

^0C	degree Celcius
cm	centimetre
g	gram
h	hour
ID	inner diameter
kg	kilogram
kJ	kilojoule
l	litre
µg	microgram
µl	microlitre
µm	micrometre
µmol	micromole
m	metre
mbar	millibar
min	minute
mg	milligram
ml	millilitre
mm	millimetre
mm^3	cubic millimetre
mM	millimolar
M	molar
nm	nanometre
rpm	revolutions per minute
%	percentage
% (v/v)	percentage, volume related to volume
% (w/v)	percentage, weight related to volume

Other Major Abbreviations

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
C	carbon
CoA	coenzyme A
DB	dry biomass
EDTA	ethylenediamine tetraacetic acid
HPLC	high performance liquid chromatography
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
TCA	tricarboxylic acid